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INTERACTION OF PARAHYDROXYBENZOIC ACID ESTERS WITH BOVINE SERUM ALBUMIN

by

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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

DEGREE OF MASTER OF SCIENCE

FACULTY OF PHARMACY

EDMONTON, ALBERTA

SEPTEMBER, 1967

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Interaction of Parahydroxybenzoic Acid Esters with Bovine Serum Albumin" submitted by Pai-Chang Sheen in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

The interaction of parahydroxybenzoic acid esters (parabens) with bovine serum albumin has been investigated by means of equilibrium dialysis, solubility and spectrophotometric techniques.

The effects of temperature and pH on the binding behavior were studied. The degree of binding increased from methyl to ethyl to propyl to butyl to hexyl to heptylparaben and the binding increased as the temperature was increased. It was found that the interactive tendency increased as the pH was raised from 4 until a maximum was achieved at pH 7.6 $\pm$ 0.2, after which a marked decrease in binding occurred. The binding constants and the number of binding sites on the protein were obtained from the dialysis data and free energy change, ΔF^{o}; enthalpy change, ΔH^{o} and entropy change, ΔS^{o} were computed. The driving force for the interaction was derived from hydrophobic bonding. The participation of other types of interaction forces are suggested also.

Correlation of the binding data with the biological activity of methyl and propylparaben was studied by an *in vitro* microbiological procedure employing *Aspergillus niger* as a test organism. It was demonstrated that the antifungal activity of methyl and propylparaben was primarily a function of free drug.

ACKNOWLEDGMENTS

I wish to express my sincere appreciation to Dr. Nagin K. Patel for his supervision and valuable assistance during the research and preparation of this thesis.

I wish to thank my parents for their encouragement.

Financial assistance from the University of Manitoba and the University of Alberta for the Teaching Assistantships; National Research Council and Smith, Kline and French Inter-American Corporation for the Research Assistantships is gratefully acknowledged.

Thanks are also extended to Miss Marguerite Ranger for her patience in the typing of this manuscript.

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INTRODUCTION

The ability of plasma proteins to reversibly bind numerous small molecules and ions has been investigated extensively (1). Most of the drugs studied have been found to interact primarily with the albumin fraction of plasma proteins (1). Although a great majority of the earlier reports were of a qualitative nature, attempts have been made in recent years to quantify the data with an ultimate aim of elucidating the nature of the equilibria and forces involved in the drug-macromolecular interaction (2-4). With the advancement of the theoretical knowledge of these interactions, considerable research has been conducted on the binding of salicylates (5,6), sulfonamides (7,8), penicillins (9), xanthines (10) and other therapeutic agents with serum albumins.

In their extensive reviews, Goldstein (1), and Brodie and Hogben (11) have discussed the importance of drug-protein association on the transport, distribution, metabolism and elimination of medicinal agents. Furthermore, there appears to be a direct relationship between the pharmacologic activity of many drugs and their ability to combine with specialized functional proteins. The investigation of the factors involved in such interactions may shed some light on the understanding of drug action.

Interactions of some benzoic acid derivatives (12) and phenol (13) with serum protein albumin have attracted much attention. Davison and Smith (6) have studied the binding of hydroxy and aminobenzoic acids with bovine serum albumin (BSA). Patel and Murray (14) demonstrated a rather high degree of binding of o-chlorobenzoic acid by BSA. Protein binding has been shown to be influenced by the degree of ionization and the size of anion (15). Thus, an anion with a fairly large nonpolar residue has imparted an increased binding capacity for BSA (15).

Both textbook and reference work (16-18) indicate a significant loss of biological activity of some antimicrobial agents in the presence of protein materials, possibly due to the binding phenomena. Aalto et al. (19) reported that antimicrobial activity of p-hydroxybenzoate esters was slightly lower in the presence of human serum. Patel and Taylor (20) found that the in vitro antifungal activity of methylparaben was primarily a function of unbound or free drug.

The purpose of this investigation was to determine the relative binding affinities between a series of p-hydroxybenzoic acid esters (parabens) and bovine serum albumin. On this basis, experiments were conducted in order to determine the following:

a) The binding constants for paraben-BSA in-

teraction for the purpose of computing various thermodynamic functions, such as free energy change, enthalpy change and entropy change of the system.

b) The influence of various hydrogen ion concentrations on the paraben-protein interaction.

Equilibrium dialysis, solubility and spectrophotometric techniques were employed to investigate the interaction of parabens with BSA.

It was also the purpose of this study to demonstrate that the antimicrobial activities of methyl and propylparaben were primarily a function of free or unbound drugs by means of an in vitro microbiological procedure.

LITERATURE REVIEW

The binding of drugs to plasma protein has attracted attention since the earliest days of chemotherapy. As early as 1904, Moore and Roaf (21,22) demonstrated that chloroform, ether and other volatile compounds were more soluble in serum than in saline solution and they suggested that this was due to interactions of these agents with the plasma proteins. In 1906, Busk (1) reported that serum changed the toxicity, diffusibility, fluorescence or light absorption and solubility of certain dyes. These phenomena he attributed to the formation of dye-albumin complexes.

Since Davis (23) postulated that the bound fraction of sulphonamide probably was devoid of bacteriostatic action, an extensive survey has been made to find correlation between the biological activity and the degree of binding. Tompsett *et al.* (24), Quinn (25) and Anton (8) found that the magnitude of reduction in antibacterial activity of penicillins and sulfadrugs caused by serum albumin was roughly in proportion to the degree of binding.

Kostenbauder and associates (26-29) have published a series of articles concerning the magnitude of intermolecular association between preservatives and several macromolecules. Employing a dialysis technique as well as solubility studies, they demonstrated that at a constant temperature, the degree of intermolecular association was a

function of both the concentration of free or unbound methylparaben and the concentration of polysorbate 80*. Pisano and Kostenbauder (27) conducted a microbiological investigation to determine the minimum inhibitory concentration of methylparaben for Aerobacter aerogenes and Aspergillus niger in control media and in media containing several concentrations of polysorbate 80. From these studies (26,27) they concluded that the preservative activity of methylparaben in the presence of polysorbate 80 was primarily a function of concentration of the unbound paraben.

Patel (30) extended the studies to correlate the binding data with the antimicrobial activity of several preservatives. He found that the antifungal activities of butylparaben, chlorocresol, propylparaben, ethylparaben, methylparaben, phenol, sorbic acid and dehydroacetic acid for Aspergillus niger, and Aerobacter Aerogenes, in the presence of macromolecules of the polyoxyethylene type, were also primarily a function of unbound preservative.

Although the binding of drug to serum protein would appear to be undesirable since drug activity is often suppressed, it can in some instances be shown

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* Polyoxyethylene (20) sorbitan monooleate. Marketed as Tween 80 by Atlas Chemical Industries, Canada, Ltd., Brantford, Canada.

to be benificial. For example, the bound drug may provide a reservoir which is converted into the active free form as needed to maintain a constant blood level over extended periods of time. Suramine, an aromatic polyamide with peripheral sulphate and carboxyl groups, strongly binds with plasma proteins. The complex acts as a reservoir to release the drug slowly and exert its anti-trypanosome action over a period of weeks or months following a single dose (31).

Substances which are capable of associating at the same site on proteins may compete with each other for binding preference (32). This phenomenon may have physiological significance. A given dose of drug would be expected to exhibit increased potency if, at the same time, another agent were also administered which would compete more strongly for the serum protein binding sites. Since the bound fraction of the drug is generally considered to be without pharmacological effect, the competition would, in effect, raise the available concentration of medicament. Kunin (32) studied the inhibitors of penicillin binding to serum proteins. Almost 250 compounds which consisted of various penicillins and their constituent groups, sulfonamides, salicylates, benzoic acid derivatives, oxazoles and other compounds were tested as serum-binding displacing agents. Penicillin G was more readily displaced than penicillin V or ancillin from serum protein.

Recently it was shown that due to the presence of bovine albumin, the antibacterial activity of a highly bound sulfonamide was increased 13-fold when phenylbutazone was present during incubation (8). This indicated that phenylbutazone displaced the bound sulfonamide from albumin.

To reach its site of action in the body, a therapeutic agent must first penetrate a succession of cellular membranes. The plasma protein and its attached drugs cannot diffuse across the membranes (33). For this reason only the unbound drug in plasma will equilibrate with drug in the protein-free fluid on the other side of the membrane. Smith et al. (34) reported that the salicylate ion was bound up to 80% in normal plasma. This extensive binding probably contributed to its slow penetration into the brain.

Numerous studies have been conducted to demonstrate the binding of therapeutic agents by serum albumin. Among the drugs which have been reported to be bound by serum albumin are tetracycline (35), rifamycin (36), novobiocin (37) and steroids (38).

Several demonstrations showed the importance of protein binding in effecting excretion with the unbound fraction of drug considered to be readily filtered through the glomeruli (39,40). Brodie and Hogben (11) have suggested that the long duration and very slow rate of metabolic degradation that characterize some drugs in

man were the result of the protective binding by serum. The rapid excretion of salicylamide correlates well with its negligible binding in contrast to the longer biological half-life of salicylic acid.

Many methods such as solubility (41-43) equilibrium dialysis (2), spectrophotometry (44-46), ultrafiltration (47) and electrophoresis (48,49) have been devised for examination of the interaction of proteins with small molecules. The most widely used procedure is the equilibrium dialysis technique. In this procedure a vessel is divided into two components by an especially prepared membrane which is impermeable to the protein molecules but fully permeable to the small molecules. If the protein is confined to one compartment and if it binds to some of the small molecules, the concentration of small molecules in the protein compartment will exceed that in the protein-free chamber. The difference between these two concentrations is a measure of the concentration of bound molecule. The usefulness of the dialysis method for demonstrating protein interaction with salts was appreciated at least a half-century ago (50).

The solubility method is suitable only with a substance of relatively limited solubility. When a solute in solution is in equilibrium with its pure phase, crystalline or liquid, its thermodynamic activity is

fixed. In a specified solvent at a fixed temperature, its concentration is also fixed. If protein is added to the solution and if some of the solute is bound by the protein, the total quantity of dissolved solute will increase to compensate for the bound quantity. The amount of bound molecule may be equated to the increase in solubility (2).

Much less frequently used than dialysis and solubility techniques is the spectrophotometric determination of small molecule-protein complexation. This method is dependent upon the detection of the disturbance to the chromophoric system of the small molecule in complexation because the bound molecules are in a different environment than in an aqueous solution (51). This disturbance is manifested by hypochromic, hyperchromic, bathochromic or hypsochromic effect in the spectra of the system. The changes in spectra may be used to calculate the extent of binding. By measuring the degree of hypochromism which increased as the concentration of protein was increased, Klotz (44,51) was the first to make the fullest use of this method to study the extent of protein-small molecule interaction. Recently, attention has been concentrated on the explanation of the reasons for spectral changes in the protein - small molecule complex. Hydrogen bonding (46,52) electrostatic effect (53) and hydrophobic bonding (54) have been proposed as the factors which contributed to

the spectra changes. This optical method is limited largely to studies of the interaction of protein with colored molecules (2). Absorption in the near ultraviolet has been utilized; however, below 300 mu, most proteins are absorbed strongly, and appropriate corrections become more difficult.

Guttman and Gadzala (46) have investigated the interaction of xanthine derivatives with bovine serum albumin. The binding was pH dependent and the structural modification of the small molecule had a pronounced influence on the degree and nature of the binding. Their results indicated that a rather specific orientation of the protein molecule was required for optimum interaction. Expansion or other changes in configuration resulting from pH or temperature changes resulted in decreased binding.

The structure of small molecules had a pronounced effect on protein binding. The binding of a series of benzoic acid derivatives had been studied by Davison and Smith (6), with the finding that the carboxylic acid group conferred primary binding capacity upon the compounds and the presence of hydrogen donors in the ortho position enhanced this capacity. Particularly interesting examples were those in which the hydrogen donor substituent was shifted from the ortho to the para position in a benzene ring. Thus o-aminoben-

zoate ion was bound more strongly by serum albumin (51) than was p-aminobenzoate. Similarly, o-hydroxyphenyl acetate showed greater affinity for albumin (55) than did the corresponding para compound. If the second substituent was not a hydrogen donor, the difference between ortho and para isomers disappeared. Thus o-chlorobenzoate and p-chlorobenzoate formed complexes with albumin of roughly equal stability (51). The greater affinity of the ortho substituted hydroxybenzoate or aminobenzoate ion probably was due to less competition between the protein and the solvent water. An intramolecular hydrogen bond is formed in these compounds so that the -OH or $-NH_2$ group does not interact strongly with solvent water molecules. On the other hand, in the corresponding para derivatives, the position of the -OH or $-NH_2$ group precludes formation of an internal hydrogen bond. Since hydrogen linkage may then form readily with water molecules, these ions would be subject to a greater attraction than would their corresponding ortho compound.

Boyer et al. studied the influence of molecular weight of the small molecules on the drug-protein interaction in case of azobenzenes to which various aliphatic side chains were attached. It was found that a smooth increase in binding occurred as the chain length was increased from butylate to caproate to caprylate to caprate.

With the large number of possible binding points

on the protein molecule, much attention has been directed toward explaining the type of intermolecular forces that might be responsible for the interactions. Serum albumin can attract small molecules by forces of association between ions, polar groups and non-polar groups and that more than one of these forces can be involved in a single combination. Any single explanation can not satisfactorily explain the binding of all small molecules and a number of factors whether alone or in combination, including hydrogen bonding (56-58), ionic attractive force (59), van der Waals forces (3,60) and hydrophobic bonding (61), play an important role in the drug-protein interactions.

Since about 40% of the total amino acids in most proteins have non-polar side chains, it might be expected that the hydrophobic bond might play an important role in drug-protein interaction (62). Very recent studies of the increased solubility of hydrocarbon gases in protein solutions over their solubility in aqueous buffers demonstrated clearly the importance of hydrophobic interaction (63). In formulating their views on hydrophobic bonding, Nemethy and Scheraga (62) indicated thermodynamic parameters to explain the transfering of various types of amino acid side chains from water to a non-polar environment. A hydrophobic bond is considered to be formed when two non-polar groups come into contact, thereby decreasing the extent of the interaction with surrounding water. This was

first pointed out by Kauzmann (64) who introduced the term hydrophobic bond to represent the attractive forces responsible for the tendency of the non-polar residues to avoid contact with the aqueous phase and to adhere to one another in the form of an intramolecular micelle.

EXPERIMENTAL

Reagents

Bovine serum albumin, fraction V (BSA), purchased from the Armour Pharmaceutical Co. (lots B24011 and D25901), was used throughout this study. The moisture content of protein was determined by drying to constant weight at 105°. A value of 3.4 per cent was found, which was employed to calculate the actual protein content. The value of 69,000 was used as the molecular weight of this protein (65). The following compounds were recrystallized from a water-ethanol mixture: methyl p-hydroxybenzoate[1], mp 125-126°; ethyl p-hydroxybenzoate[1], mp 115-116°; propyl p-hydroxybenzoate[1], mp 95-96°; butyl p-hydroxybenzoate[1]; mp 68-69°; hexyl p-hydroxybenzoate[1] mp 51-52°; and heptyl p-hydroxybenzoate[1] 47-48°. Reagent grade acetic acid[2], sodium acetate[2], monobasic potassium phosphate[2], dibasic potassium phosphate[2], boric acid[2], sodium borate[2] and potassium chloride[3], dextrose[4] (anhydrous), sodium chloride[3], magnesium sulfate[3], ammonium dihydrogen phosphate[4] were used to prepare the buffer solutions or culture medium. All paraben and protein solutions were prepared immediately prior to use.

[1]Methyl, ethyl, propyl, butyl, hexyl, and heptyl parasept, purified, Tenneco Chemicals, Inc., New York, N.Y.

[2]Allied Chemical, New York, N.Y.

[3]The McArthur Chemical Co., Montreal, Quebec.

[4]British Drug Houses Laboratory Reagent.

Equilibrium Dialysis Studies

The general approach and technique employed in this study were essentially the same as those employed by Patel and Foss (66), in studying the interaction of pharmaceuticals with macromolecules, with the exception that seamless cellulose membranes[5] were employed in the present work. The membranes were placed in water and heated to 90° for one hour, then washed with distilled water several times in order to remove contaminating substances in the cellophane (8).

The dialysis cells consisted of two Plexiglass[6] blocks measuring 7.6 by 7.6 by 1.9 cm with each half containing a cylindrical cavity of 12 ml capacity, 3.4 cm internal diameter and 1.3 cm depth. Access to the cavity was provided by threaded Plexiglass plugs. The cells were assembled by placing a small square of cellophane membrane approximately 4 by 4 cm over the cavity and then clamping the two blocks together with stainless steel head bolts and wing nuts. Ten millilitres of appropriate solutions were then pipetted into each cavity and the stoppers fitted with O-rings[7] were

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[5]Dialyzer Tubing, Fisher Scientific Co., Fair Lawn, N.J.

[6]Methyl Methacrylate, Rohm and Haas Co., Philadelphia, Pa.

[7]National O-Rings, Division of Federal Mobile Service, Detroit, Michigan.

screwed tightly. The cells were then agitated in an Aminco water bath[8], equipped with a rotational shaker*, at a desired temperature, ± 0.1°, until equilibrium was established. Time sufficient to ensure equilibrium was ascertained by placing a control cell which contained a buffer solution in place of the protein solution. An identical concentration of the drug on both sides of the membrane was indicative of equilibrium. Equilibrium was established at the end of 16 hours in all cases.

Assymetry in the distribution of the small molecule may be obtained due to the Donnan effect for which an ionic strength of 0.16 is large enough to render this effect negligible (67). Thus all the buffer solutions were adjusted to a value of 0.16 by addition of KCl.

Study at Different Temperatures. Because the pH of plasma is 7.4, the experiments were conducted at pH 7.4 using phosphate buffer. The temperatures of 30.0° for all parabens, also 10.0° for methylparaben and 20.0° for ethyl, propyl, and butylparaben were used. For most of the experiments, a protein concentration of 1.0×10^{-4} M was used.

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[8] Constant Temperature Laboratory Bath, American Instrument Co. Inc., Silver Spring, Md.

* Designed by Dr. N.K. Patel and manufactured by the Machine Shop, University of Alberta.

In addition, a protein concentration of 2.5×10^{-4} M was also used for propylparaben.

Study at pH 6.6. This part of the binding data was collected at pH 6.6 and at 30° to approximate conditions utilized in a previous microbiological study (30), since it was the object of this experiment to correlate the binding data with the microbiological work. The initial concentration ranges of methyl and propylparaben were selected on the basis of obtaining free antimicrobials comparable to their inhibitory concentrations in the presence of 0.1 mM BSA.

Binding of Propylparaben with Various Concentrations of BSA. The experiments were conducted at pH 7.4 and 30.0°. An acetate buffer was employed for the pH range of 4 to 5; phosphate buffer for pH 6 to 8; borate buffer for pH 8 to 9. The initial molar concentrations of methyl, ethyl, propyl, and butylparaben were 2.63×10^{-3}, 2.41×10^{-3}, 2.22×10^{-3} respectively.

Solubility Studies.

Due to the low water solubilities of hexyl and heptylparaben, the solubility method was used to evaluate the interaction between these parabens and BSA.

Excess quantities (0.1 g) of parabens were placed in 15 ml vials containing 10 ml of varying concentration of BSA solutions. A control vial was included, differing from

the others in having 10 ml of buffer solution instead of the protein solution. The vials were sealed with aluminum foil, and closed tightly with the screw caps. They were agitated in the water bath at 30.0° for a period of 24 hours. At the end of this period, the vials were allowed to stand for 1 hour in the water bath to settle down the excess of parabens before carrying out the analytical procedure.

Spectrophotometric Studies

Spectrophotometric examination of paraben-containing solutions in the absence and presence of BSA were made on a Hilger Ultrascan recording spectrophotometer, model H999. For each solution that contained paraben and protein, a corresponding solution containing an equivalent concentration of protein was prepared. The latter solution was used as a reference blank in the spectrophotometric examination of the sample and served to blank out the absorbance contribution due to the protein. Varying concentrations of BSA from 0.05 mM to 0.1 mM were used. The concentration of paraben used was 6.4×10^{-5} M which had an absorbance reading around 0.8 in the absence of BSA. The measurements were conducted at room temperature and at pH 7.4.

Analytical Methods

At the end of equilibrium time, 1 or 2 ml aliquots were removed from both sides of the membrane and proper dilutions were made with 0.01 N HCl to suppress the dissociation of parabens. The degree of dilution depended

on the concentration of the drug and protein solution to yield an absorbance reading below 0.800. The parabens were assayed using a Beckman spectrophotometer, model DU, at a wavelength of their maximum absorbance, 256 mμ. Any interference due to the protein was eliminated by using the appropriate concentration in the reference cell and it was observed that at the very high dilutions employed for the analysis of parabens, there was no effect on the absorbance of parabens in the presence of BSA. Any interference caused by dialyzable components in BSA was assumed to be negligible because of the high dilutions required in a spectrophotometric assay. The pH of the solutions in the dialysis studies was recorded at the end of the experiment, with no appreciable change noted. All the pH measurements were made with a Beckman zeromatic pH meter, model 76, using a combination electrode.

In the case of solubility studies, the general assay procedure was the same as that used in the equilibrium dialysis method, except 1 or 2 ml aliquots were withdrawn by means of a pipette, the tip of which was covered by fine glasswool to exclude the solid paraben.

Microbiological Studies

Microorganism A filamentous fungus Aspergillus niger[9] was selected as the principle microbe, because it

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[9] UAMH No. 1456, obtained from Provincial Laboratory of Public Health, Edmonton, Alberta.

grows luxuriously in liquid media, produces readily visible mycelium, and sporulates easily.

Culture Medium. To avoid any competing interactions between parabens and the components of common bacteriological media containing protein material such as beef extract, the following simple, chemically defined synthesis culture medium as employed by Pisano and Kostenbauder (27) was utilized:

Dextrose, anhydrous	5.00%
NaCl	0.50%
$MgSO_4.7H_2O$	0.02%
$K_2HPO_4.3H_2O$	0.10%
$NH_4H_2PO_4$	0.10%
Distilled water, to make pH of medium 6.6	100 %

Preparation of Test Media and Cultures. A 0.2% solution of methylparaben was prepared in culture medium and desired concentrations of methylparaben containing 0.69% BSA were made by the following procedure. Appropriate amounts of 0.2% methylparaben solution and culture medium were pipetted into a 18 x 150 mm test tube, in duplicate to produce various concentrations of methylparaben. The test tubes were stoppered with polypropylene caps[10], and sterilized by autoclaving at 15 pounds pressure for 15 minutes. A 3.45% of BSA solution was prepared in culture medium and sterilized by millipore filtration using a hypodermic syringe, Swinney adapter and millipore Ha[11],

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[10]Bacti-capall, Fisher Scientific Co., Fair Lawn, N.J.

[11]Cellulose membrane, marketed as Millipore Ha, Millipore Filter Co., Bedford, Mass.

because proteins would be denatured at the high temperature of autoclaving. Two ml of this sterilized BSA solution was transferred aseptically to each of the above test tubes.

Due to the low solubility of propylparaben, a 0.05% stock solution of propylparaben in culture medium was prepared. The required concentrations of propylparaben containing 0.69% BSA were made by adding a weighed amount of BSA to each solution. Each solution was sterilized by millipore filtration. Ten ml of the sterilized solution was transferred aseptically to an 18 x 150 mm test tube in duplicate for each concentration.

The possibility of degradation of methylparaben was investigated by determining its ultraviolet absorbance before and after autoclaving. No change was observed in the absorbance characteristic. Similar determinations were made on methyl and propylparaben solution after two weeks' storage at 30.0° with no change observed. It was assumed that the compounds studied were reasonably stable under the experimental conditions.

Aspergillus niger* was cultured on slants of Sabouraud's dextrose agar for 3 days. A suspension was prepared by inoculating approximately 3 ml of the sterile culture medium with 2 loopsful of the fungus spores. Each of the test tubes was inoculated with a loopful of the spore

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*It was kindly prepared by Miss Margorie Dineen of the Provincial Laboratory of Public Health, Edmonton, Alberta.

suspension. The cultures were incubated at 30°* and the growths in the form of mycelial mats were observed visually each day for one week.

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* This temperature was selected because the dialysis studies were conducted at 30°.

THEORY AND RESULTS

Theory of Multiple Equilibrium

Klotz (2) first applied the law of mass action to develop the equation of association between small molecules and proteins.

Single Association. According to the law of mass action where one mole of small molecule of the type A associate with one mole of protein P, to form a simple complex PA, the equilibrium can be described as:

$$P + A \rightleftharpoons PA \qquad \text{(Eq.1)}$$

The association or binding constant, k, is then defined by the equation

$$k = \frac{(PA)}{(P)\ (A)} \qquad \text{(Eq.2)}$$

in which the quantities in parenthesis represent the molar concentration of the respective species.

The total protein concentration can be represented by means of the relation

$$(Pt) = (PA) + (P) \qquad \text{(Eq.3)}$$

Rearranging and then substituting Eq. 3 into Eq. 2 gives

$$\frac{(PA)}{(Pt)} = \frac{k(A)}{1 + k(A)} \qquad \text{(Eq.4)}$$

The ratio (PA/(Pt) is evidently the number of moles of bound A per mole of total protein, and is designated by the symbol r, thus

$$r = \frac{k(A)}{1 + k(A)} \qquad \text{(Eq.5)}$$

A simple linear relation is obtained by transposition of Eq. 5 to the form

$$\frac{r}{(A)} = k - rk \qquad \text{(Eq.6)}$$

Multiple Association. Practically all protein combinations produce complexes with the components present in molecular quantities other than in a simple ratio of one to one. The complex formation can be considered to be a stepwise procedure (2).

$$\begin{aligned} P + A &\rightleftharpoons PA \\ PA + A &\rightleftharpoons PA_2 \\ PAn\text{-}1 + A &\rightleftharpoons PAn \end{aligned} \qquad \text{(Eq.7)}$$

where the n is the maximum number of moles of small molecule which can be bound per mole of protein. The ultimate equation representing the multiple association is

$$\frac{nk(A)}{1 + k(A)} = r \qquad \text{(Eq.8)}$$

The value of n and k can be graphically determined by plotting the data in accordance with Eq. 9, which represents a rearranged form of Eq. 8.

$$\frac{r}{(A)} = nk\text{-}rk \qquad \text{(Eq.9)}$$

A plot of r/(A) vs. r will result in a straight line of slope -k and ordinate and abscissa intercepts of nk and n respectively (68). This equation has been employed in the current study to determine the values of k and n for the paraben-protein interaction.

TABLE I

A Typical Calculation from Dialysis Studies

DRUG SIDE		BSA SIDE
	Before Dialysis	
Volume	10.0 ml	10.0 ml
Paraben Concn.	0.05% $=2.81 \times 10^{-3}$ M	
BSA Concn.		1.0×10^{-4} M
pH	7.4	7.4
	After Dialysis	
Volume	10.0 ml	10.0 ml
Paraben Concn.	9.49×10^{-4} M	18.6×10^{-4} M
BSA Concn.		1.0×10^{-4} M
pH	7.4	7.4

Total number of moles of paraben bound in albumin side

$$\frac{1}{100}\left(18.6 \times 10^{-4} - 9.49 \times 10^{-4}\right) = 9.11 \times 10^{-6}$$

Total albumin in albumin side 1.0×10^{-6}

$$r = \frac{\text{moles of paraben bound}}{\text{total moles of albumin}} = \frac{9.11 \times 10^{-6}}{1.0 \times 10^{-6}} = 9.11$$

(A) = concentration of unbound paraben = 9.49×10^{-4} M

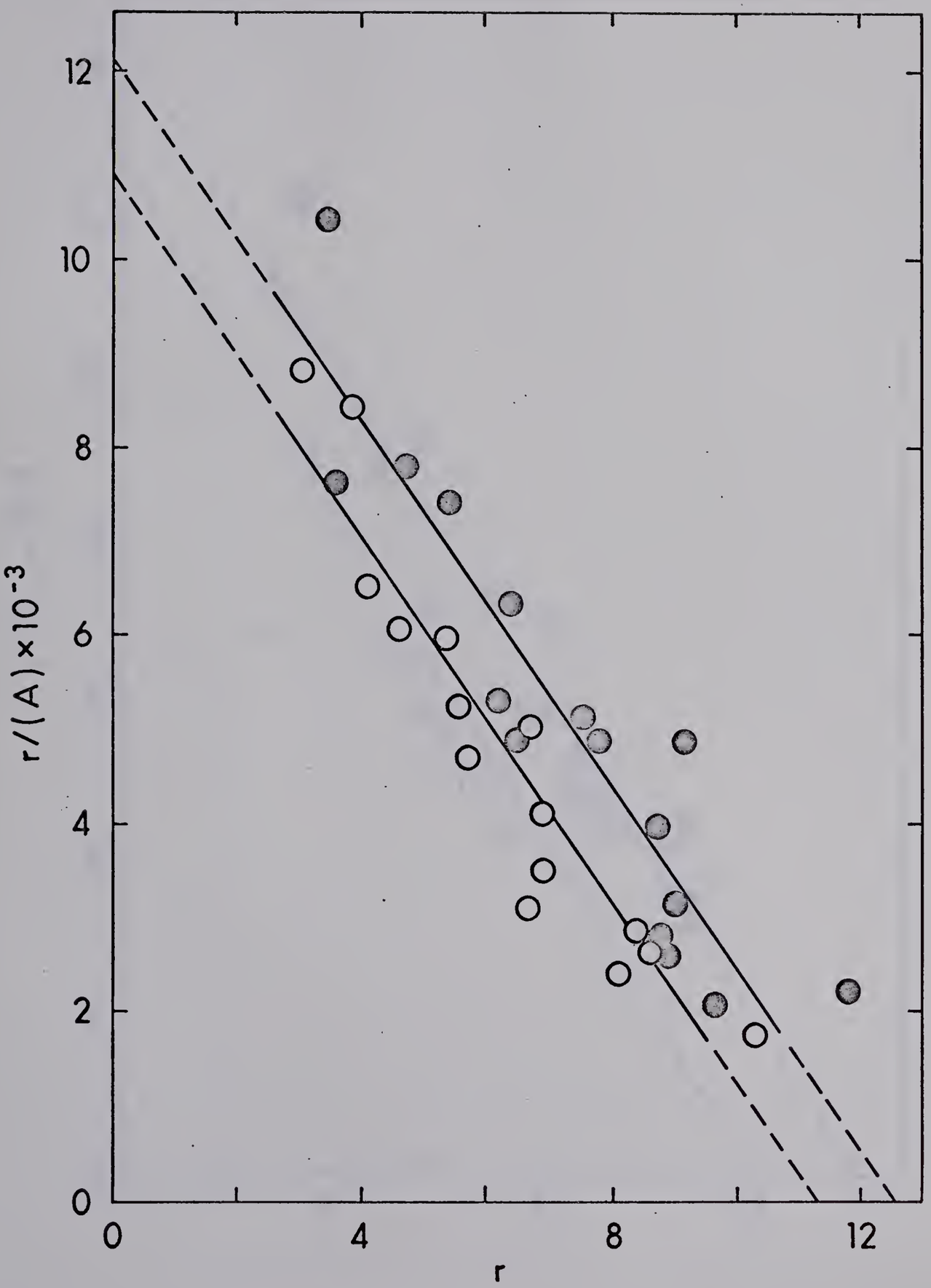

FIG. 1. THE BINDING OF METHYLPARABEN BY 0.1mM BOVINE SERUM ALBUMIN AT pH 7.4.
KEY: ○, at 30°, ● , at 10°.

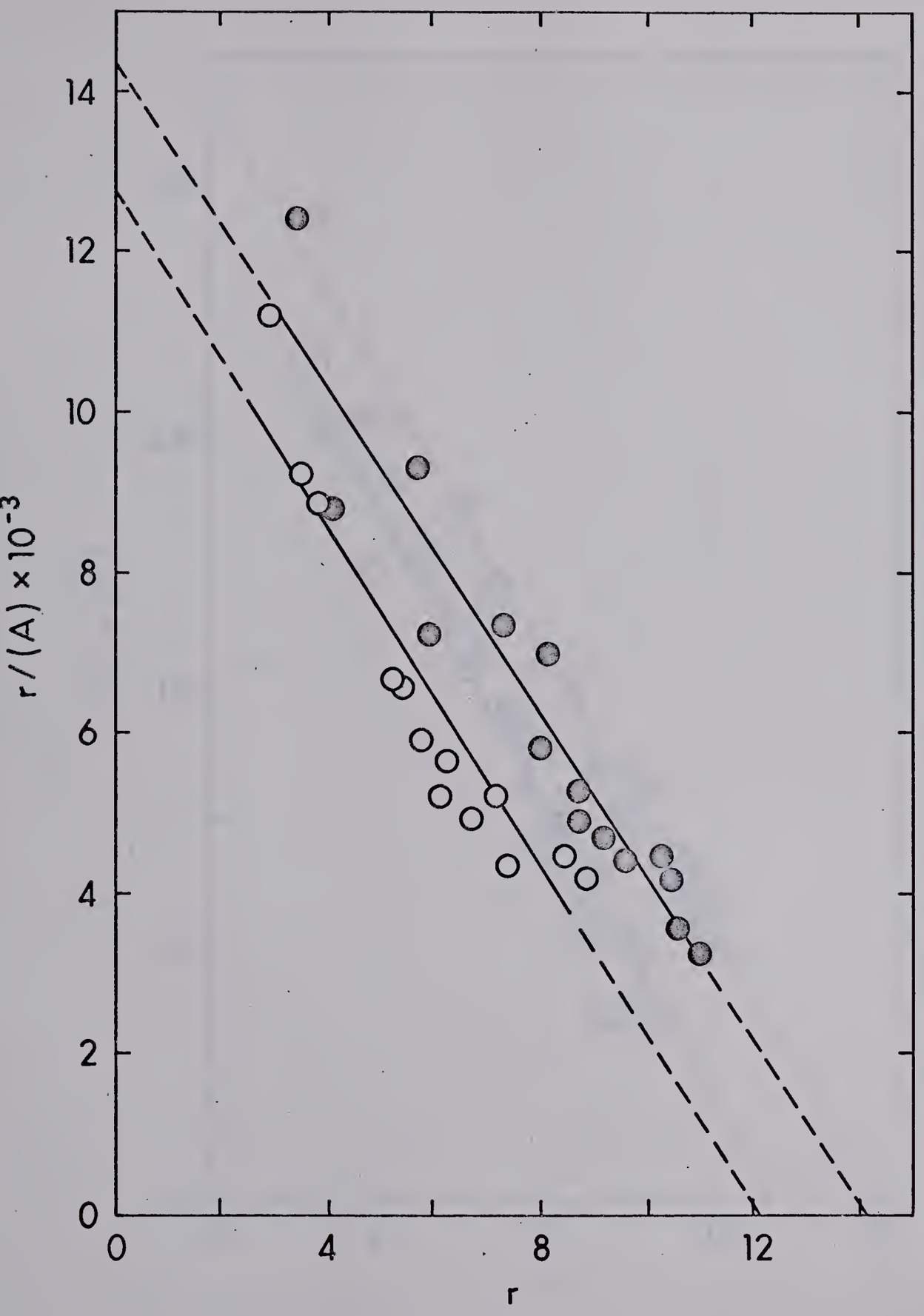

FIG. 2. THE BINDING OF ETHYLPARABEN BY 0.1 mM BOVINE SERUM ALBUMIN AT pH 7.4.
KEY: ○, at 30°; ●, at 20°.

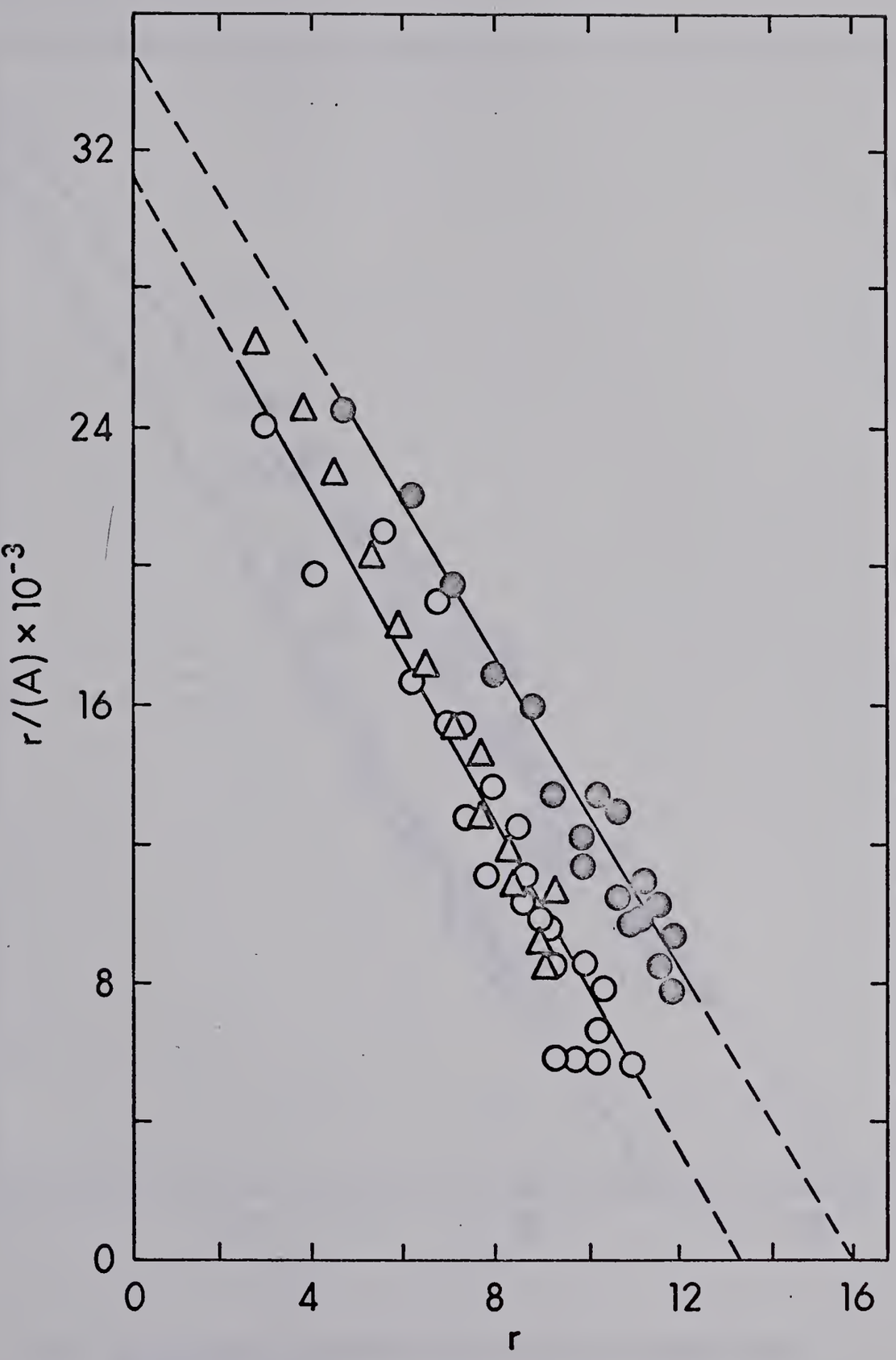

FIG. 3. THE BINDING OF PROPYLPARABEN BY BOVINE SERUM ALBUMIN AT $_{p}$H 7.4.
KEY: ○, at 30°, 0.1mMBSA; △, at 30°, 0.25mMBSA;
●, at 20°, 0.1mMBSA.

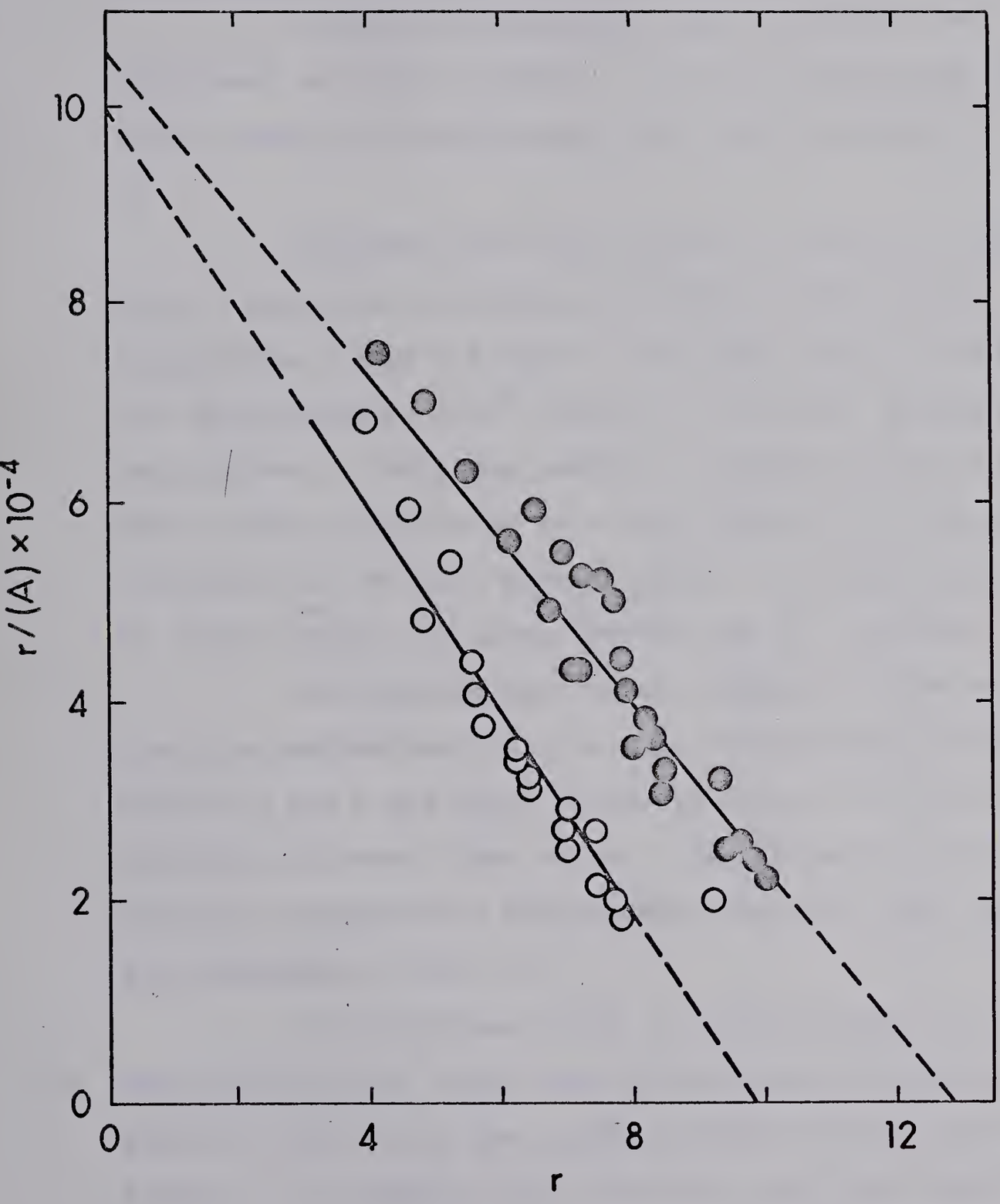

FIG. 4. THE BINDING OF BUTYLPARABEN BY 0.1 mM BOVINE SERUM ALBUMIN AT pH 7.4.
KEY: ○, at 30°; ●, at 20°.

Equilibrium Dialysis Studies of the Paraben-Protein Interaction

A typical calculation from the results of a dialysis experiment is shown in Table I, and the binding data for methyl, ethyl, propyl and butylparaben are listed in Table II through X.

Figures 1 to 4 are plotted in terms of r/(A) vs. r. These illustrate the binding of methyl, ethyl, propyl, and butylparaben at pH 7.4 and at two temperatures, 10.0° and 30.0° for methylparaben; 20.0° and 30.0° for ethyl, propyl and butylparaben. The plots result in straight lines in all cases with a slope value of -k and ordinate and abscissa intercepts of nk and n respectively. The graphs were drawn by least squares fit using IBM7040 and 360 computers.

The binding data of all parabens follow Eq. 8. It should be emphasized that there is a reciprocal relationship between n and k and small change in slope of the line will markedly influence these values. The values of n and k at different temperatures evaluated by the plot r/(A) vs. r are presented in Table XI.

In the present work, a concentration of 0.25 mM BSA was also used in the case of the protein-propylparaben system. The results are given in Table VIII and plotted in Figure 3. It appears from this plot that there was no detectable protein concentration effect with respect to the relation between r and (A) in the binding of propylparaben with BSA.

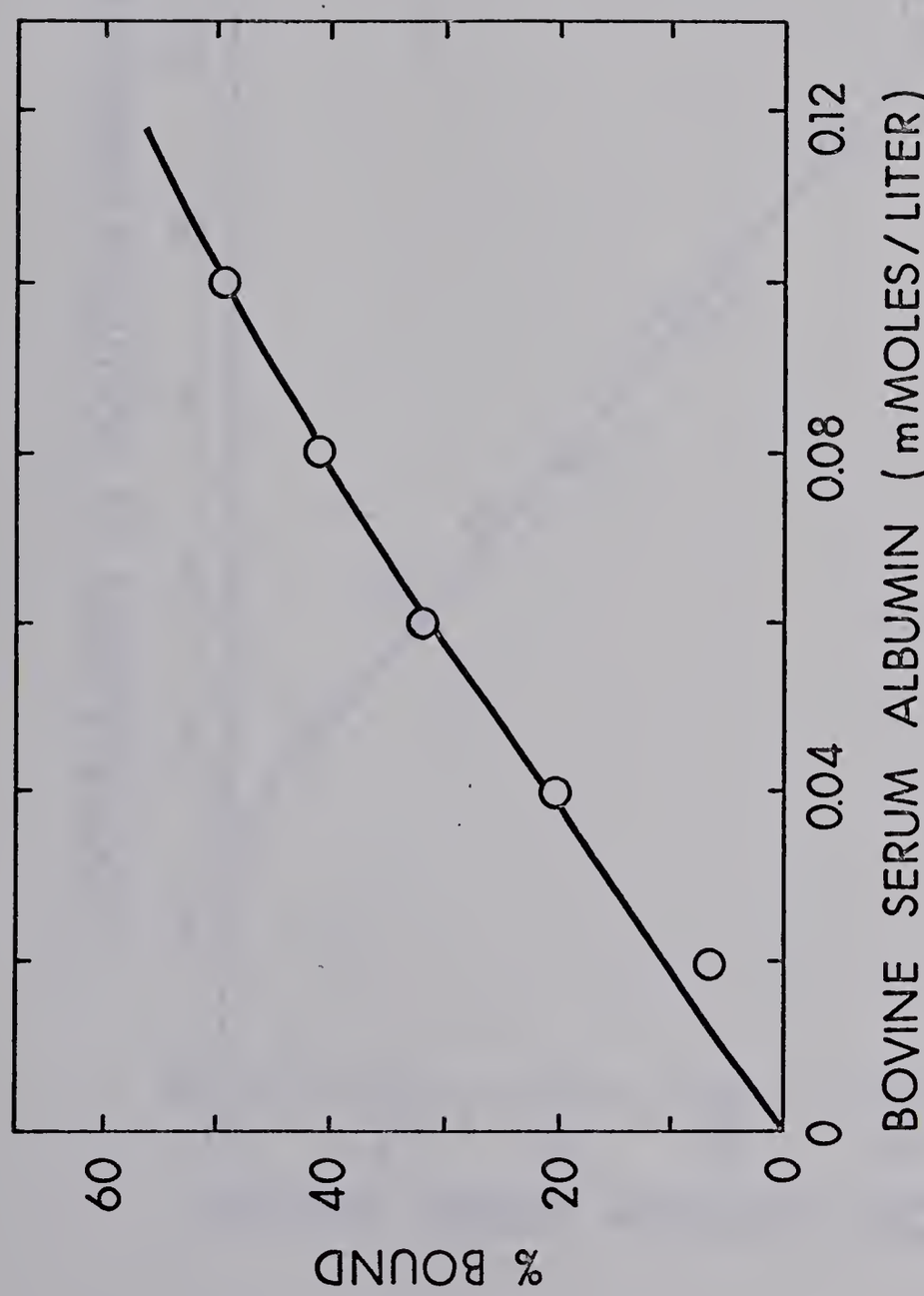

FIG. 5. PERCENTAGE BINDING OF PROPYLPARABEN TO BOVINE SERUM ALBUMIN OF VARIOUS CONCENTRATION AT pH 7.4 AND AT 30°.

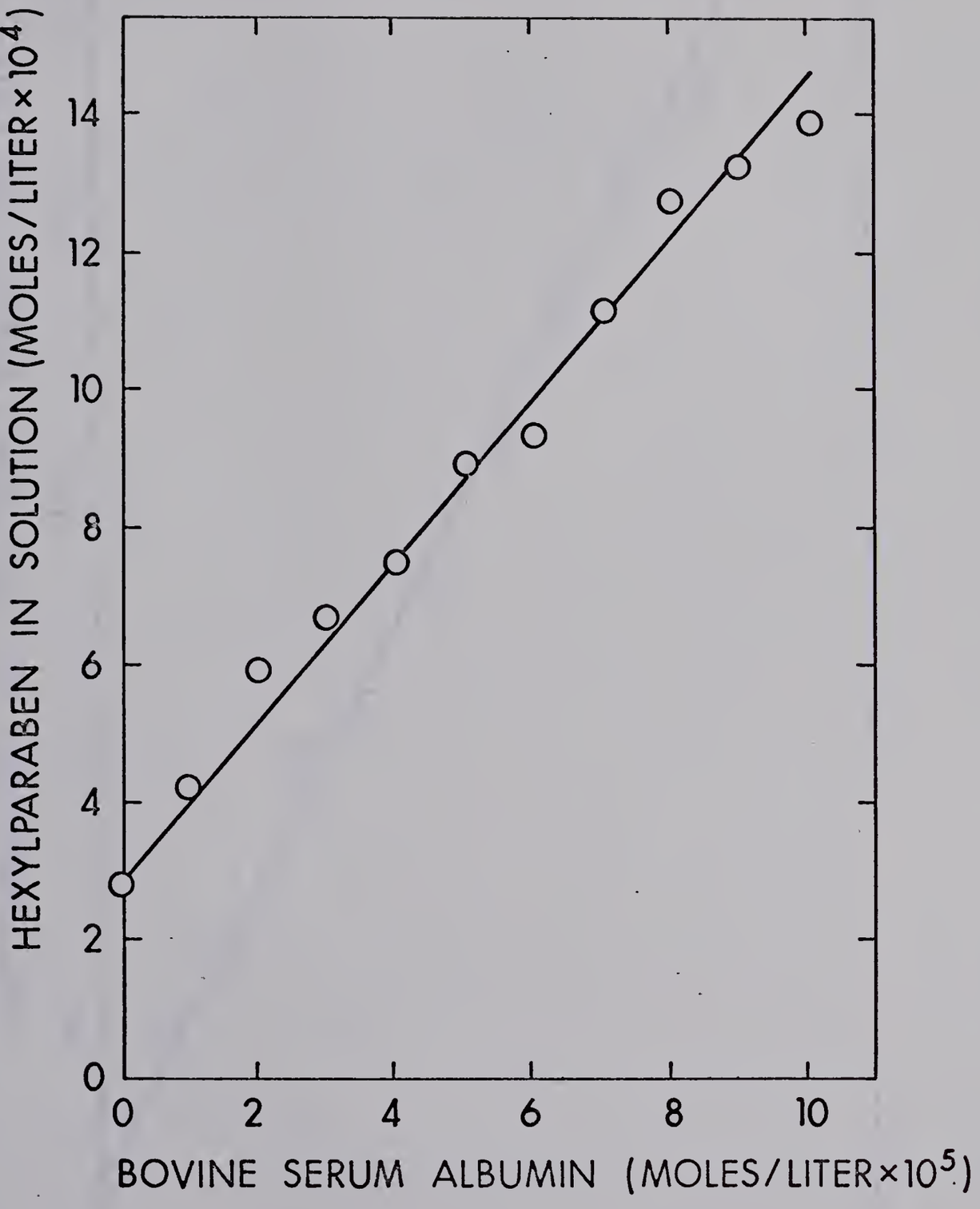

FIG. 6. THE SOLUBILITY OF HEXYLPARABEN IN VARIOUS CONCENTRATIONS OF BOVINE SERUM ALBUMIN AT pH 7.4 AND AT 30°.

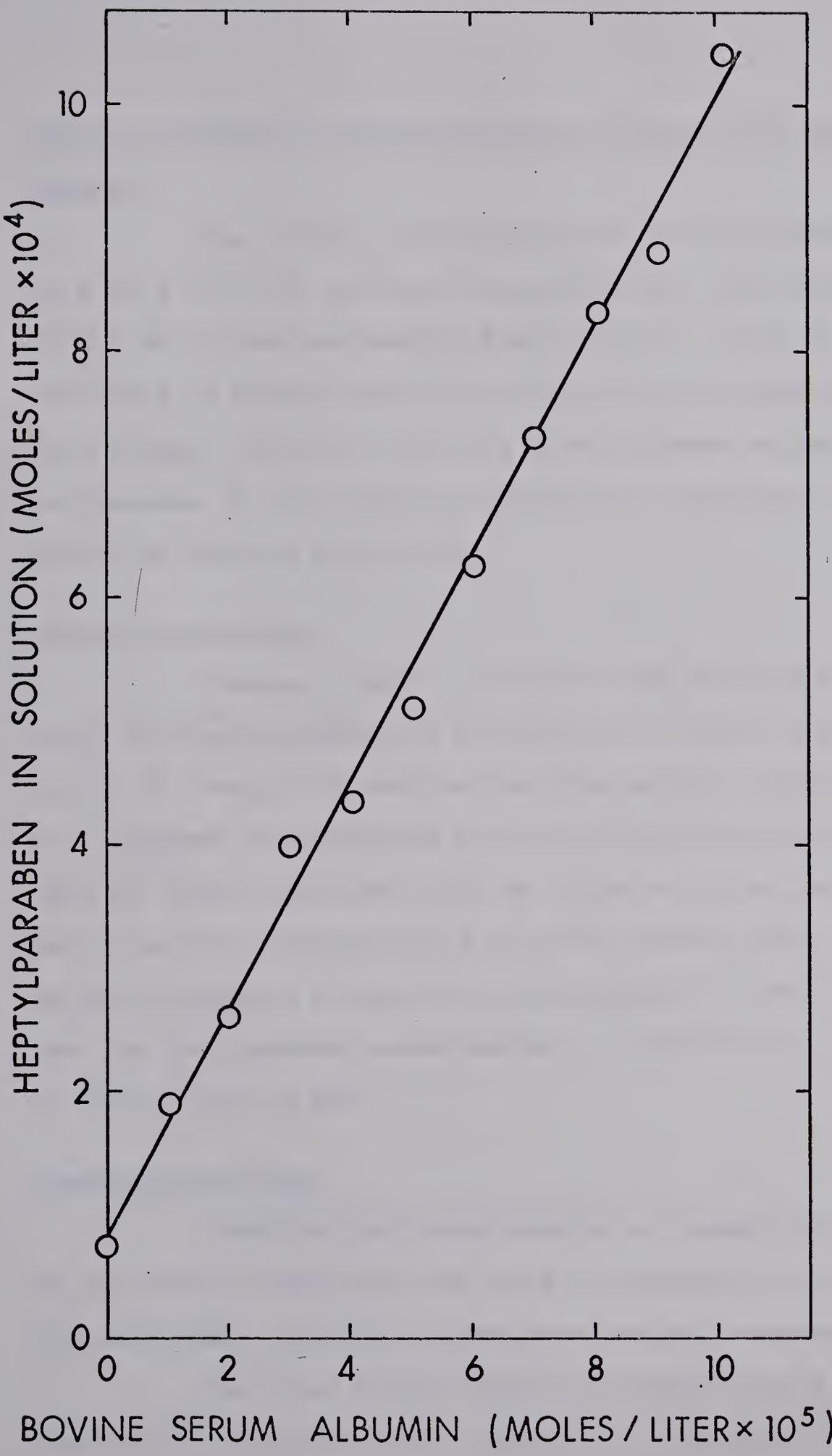

FIG. 7. THE SOLUBILITY OF HEPTYLPARABEN AT VARIOUS CONCENTRATIONS OF BOVINE SERUM ALBUMIN AT pH 7.4 AND AT 30°.

Degree of Binding by Various Concentrations of Bovine Serum Albumin

The binding of propylparaben in the concentration of 2.63×10^{-3} M to various concentrations of BSA from 0.02 mM to 0.1 mM is demonstrated in Table XII and Figure 5, and was found to range from 6.53 to 49.5% of the bound drug. This figure indicates that at a given paraben concentration, an increase in the albumin concentration increases the degree of binding to protein.

Solubility Studies

Figures 6 and 7 illustrate the solubilities of hexyl and heptylparaben as a function of albumin concentration. In solubility studies the free paraben concentrations were assumed to correspond with the solubilities of the parabens in buffer solutions (26) at definite pH and temperature; and the concentrations of bound paraben were obtained as the difference between the total paraben in solutions and the free paraben concentrations. The data are displayed in Table XIII and XIV.

Thermodynamic Data

Quantitative investigation of temperature effects on equilibrium complexes can lead to information on the thermodynamic properties of paraben-protein complexation (69).

The free energy change of interaction, ΔF^{o}, is related to the association constant k of paraben-protein complex by the relationship

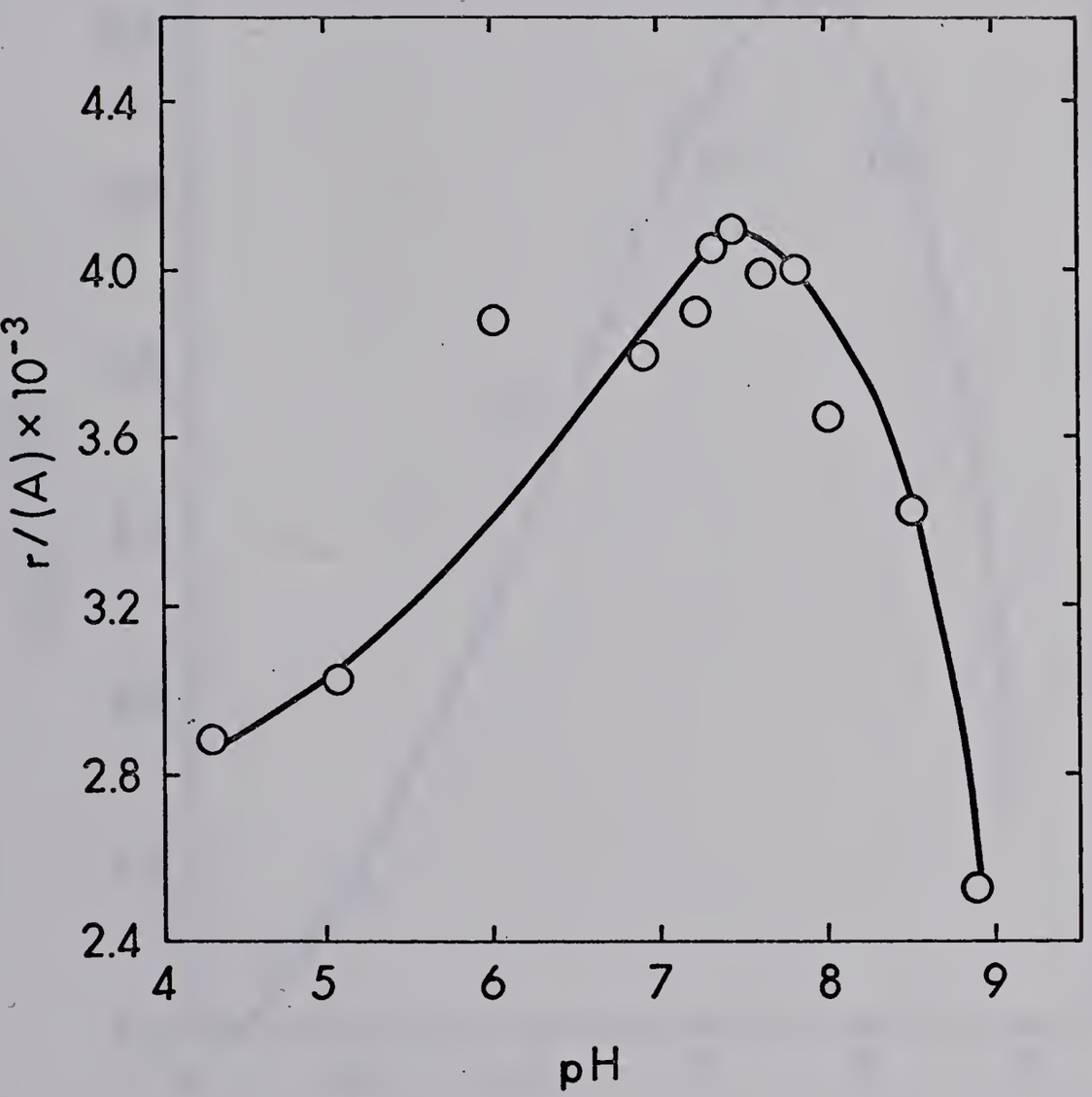

FIG. 8. THE BINDING OF METHYLPARABEN BY BOVINE SERUM ALBUMIN AS A FUNCTION OF pH AT 30°.

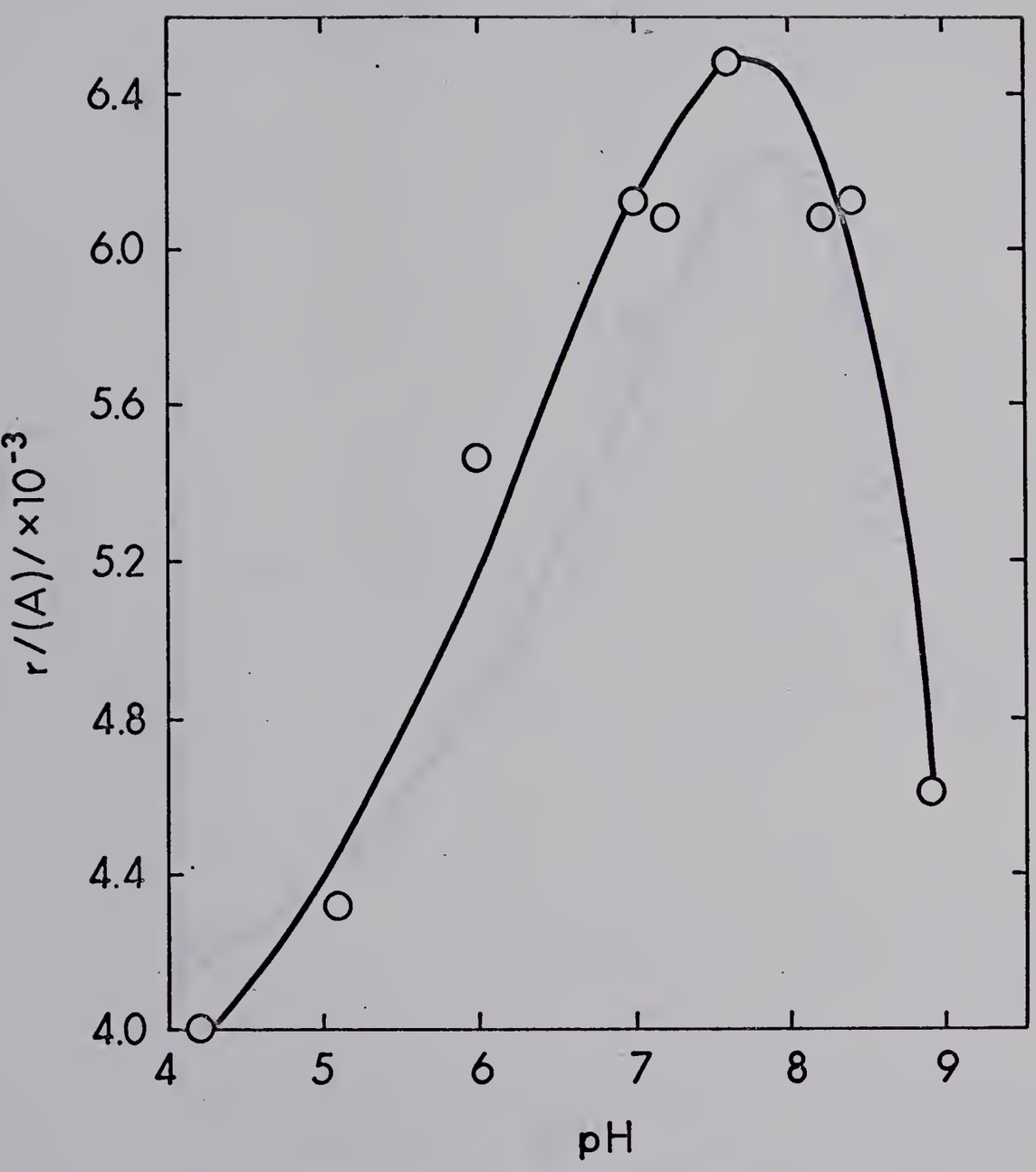

FIG. 9. THE BINDING OF ETHYLPARABEN BY BOVINE SERUM ALBUMIN AS A FUNCTION OF pH AT 30°.

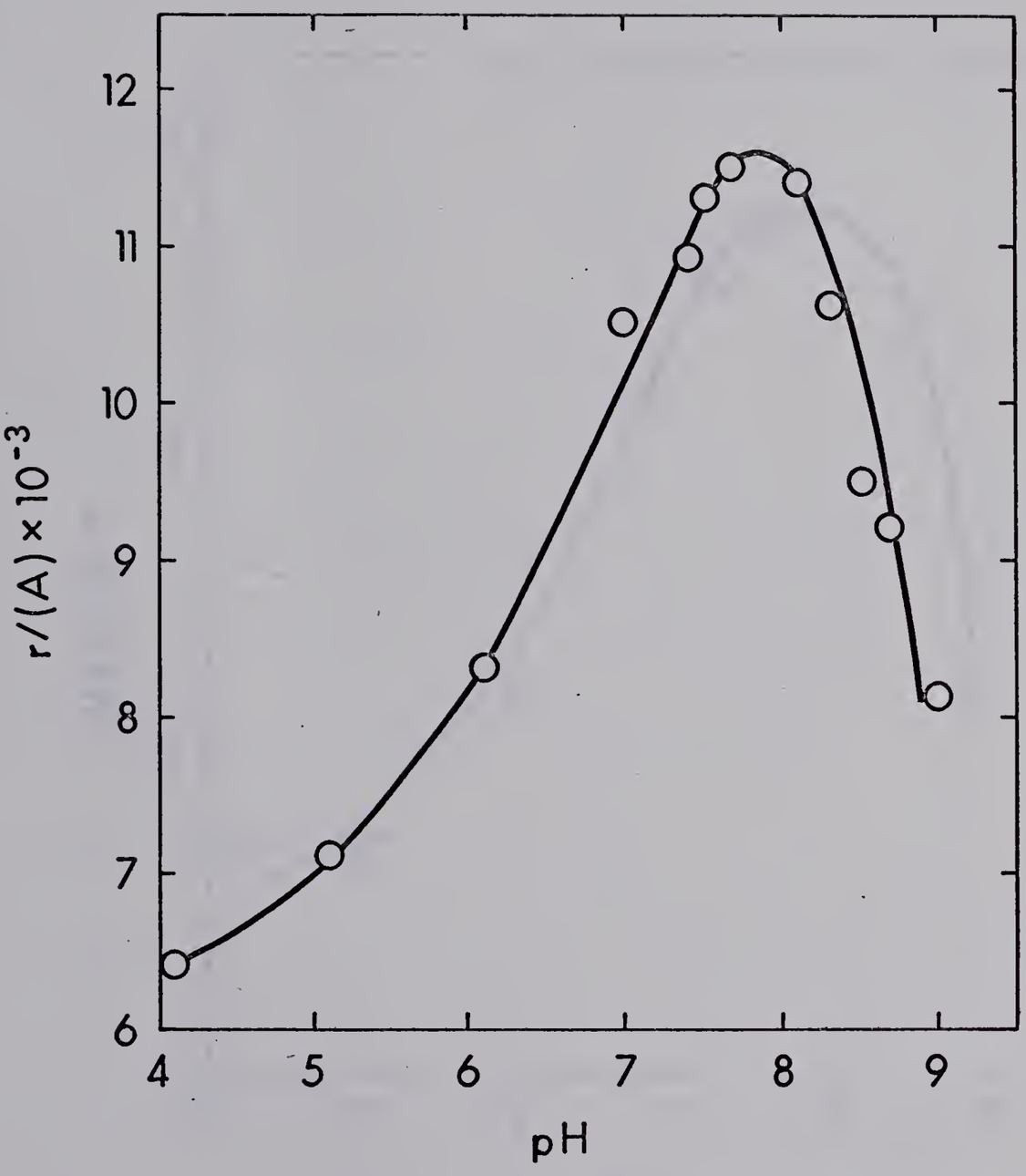

FIG. 10. THE BINDING OF PROPYLPARABEN BY BOVINE SERUM ALBUMIN AS A FUNCTION OF $_{p}$H AT 30°.

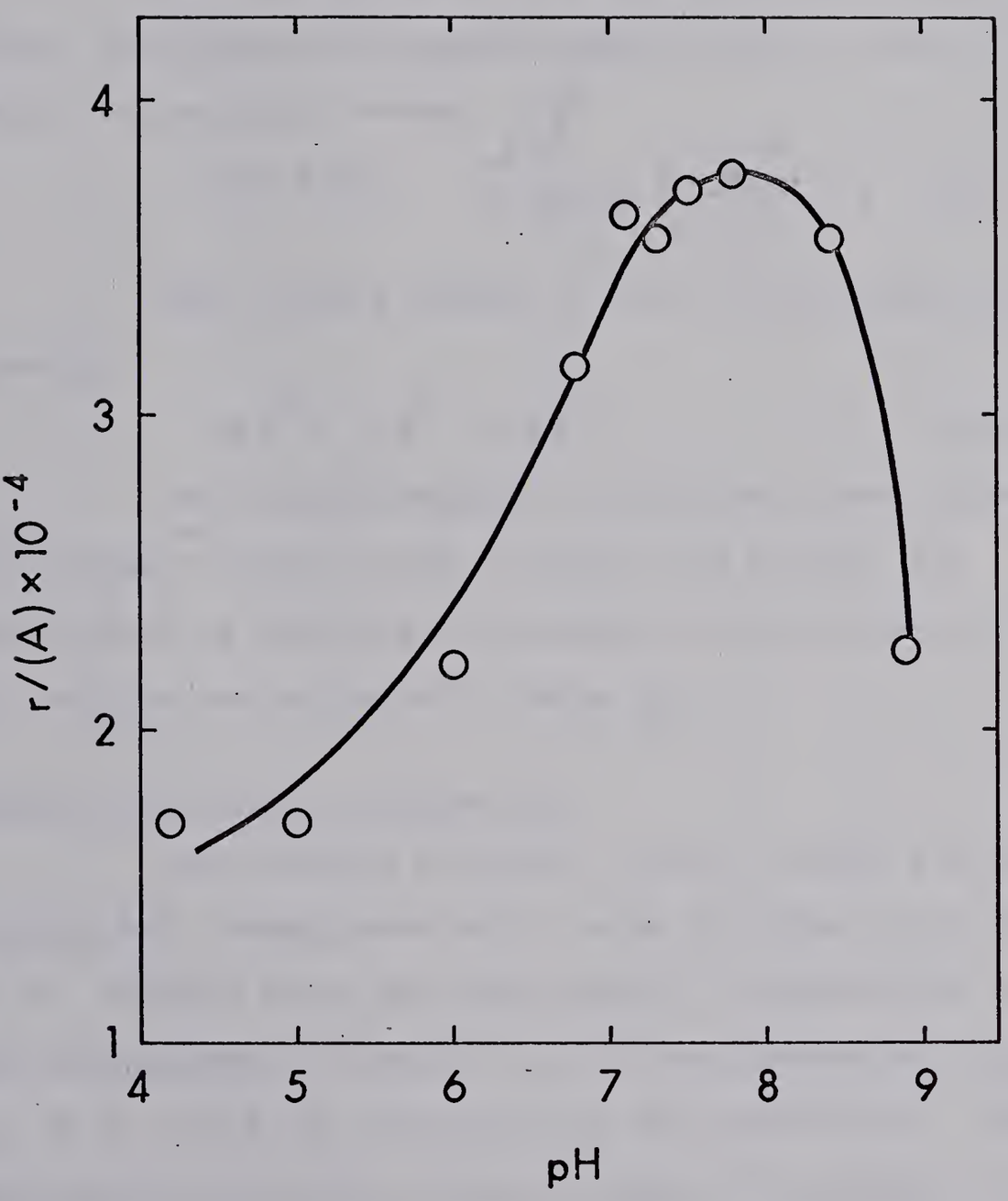

FIG. 11. THE BINDING OF BUTYLPARABEN BY BOVINE SERUM ALBUMIN AS A FUNCTION OF pH AT 30°.

$$\Delta F^{\circ} = -2.303\ RT \log k \qquad \text{(Eq.10)}$$

where R represents the gas constant and T the absolute temperature.

When the values of k at two temperatures are known, the Clausius-Clapeyron equation may be used to obtain the enthalpy change, ΔH°

$$\log k_2/k_1 = \frac{\Delta H^{\circ}}{2.303\ R}\left(\frac{T_2 - T_1}{T_1 T_2}\right) \qquad \text{(Eq.11)}$$

The entropy change S° can be obtained from equation

$$\Delta F^{\circ} = \Delta H^{\circ} - T\Delta S^{\circ} \qquad \text{(Eq.12)}$$

By the thermodynamic method mentioned above, the change in free energy, enthalpy and entropy for the binding of one mole of paraben was calculated and the results are depicted in Table XI.

Effect of pH on the Interaction

The binding of methyl, ethyl, propyl and butyl-paraben was investigated over a wide pH range, from pH 4 to 9. In each study the same initial concentration of paraben was employed. Figures 8 to 11 are plotted as r/(A) vs. pH to yield the pH profile of the interaction. The experimental data are listed in Table XV to XVIII. It is seen that essentially parabens exhibited an increased magnitude of binding as the pH was raised from 4 until a maximum binding was achieved at 7.6 $\pm$ 0.2 above which a marked decrease in binding occurred.

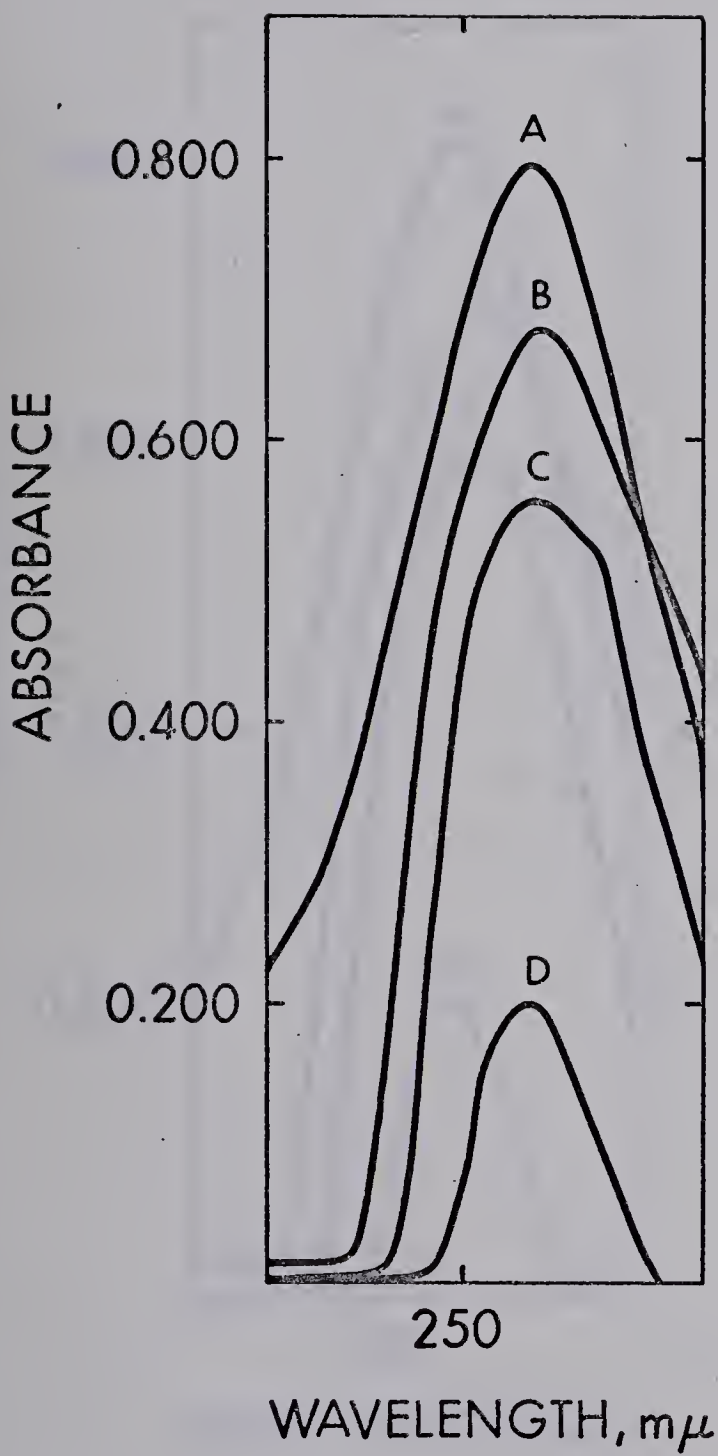

FIG. 12. THE INFLUENCE OF BOVINE SERUM ALBUMIN ON THE SPECTRUM OF METHYLPARABEN AT pH 7.4.

KEY: A, no BSA; B, 0.025 mMBSA; C, 0.05 mMBSA; D, 0.1 mMBSA.

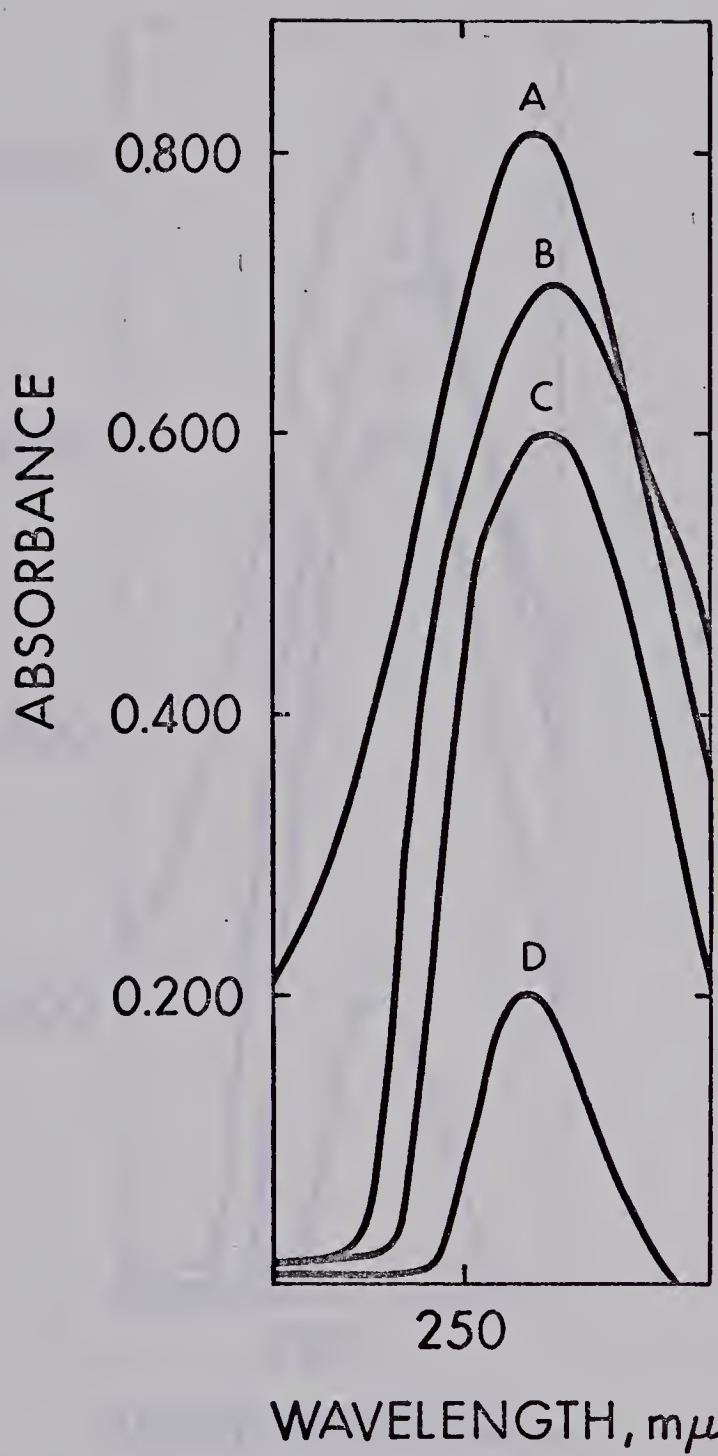

FIG. 13. THE INFLUENCE OF BOVINE SERUM ALBUMIN ON THE SPECTRUM OF ETHYLPARABEN AT pH 7.4.

KEY: A, no BSA; B, 0.025 mMBSA; C, 0.05 mMBSA; D, 0.1 mMBSA.

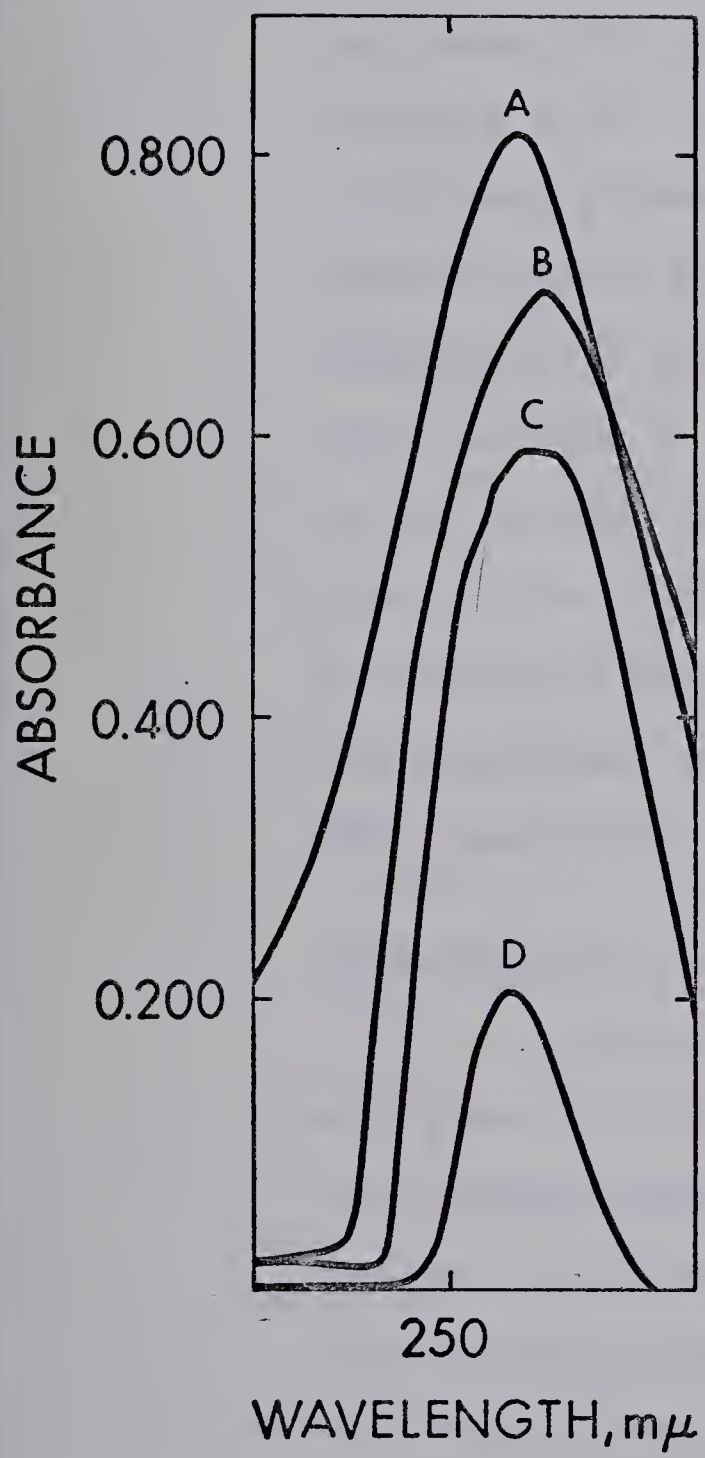

FIG. 14. THE INFLUENCE OF BOVINE SERUM ALBUMIN ON THE SPECTRUM OF PROPYLPARABEN AT pH 7.4.

KEY: A, no BSA; B, 0.025mMBSA; C, 0.05mMBSA: D, 0.1mMBSA.

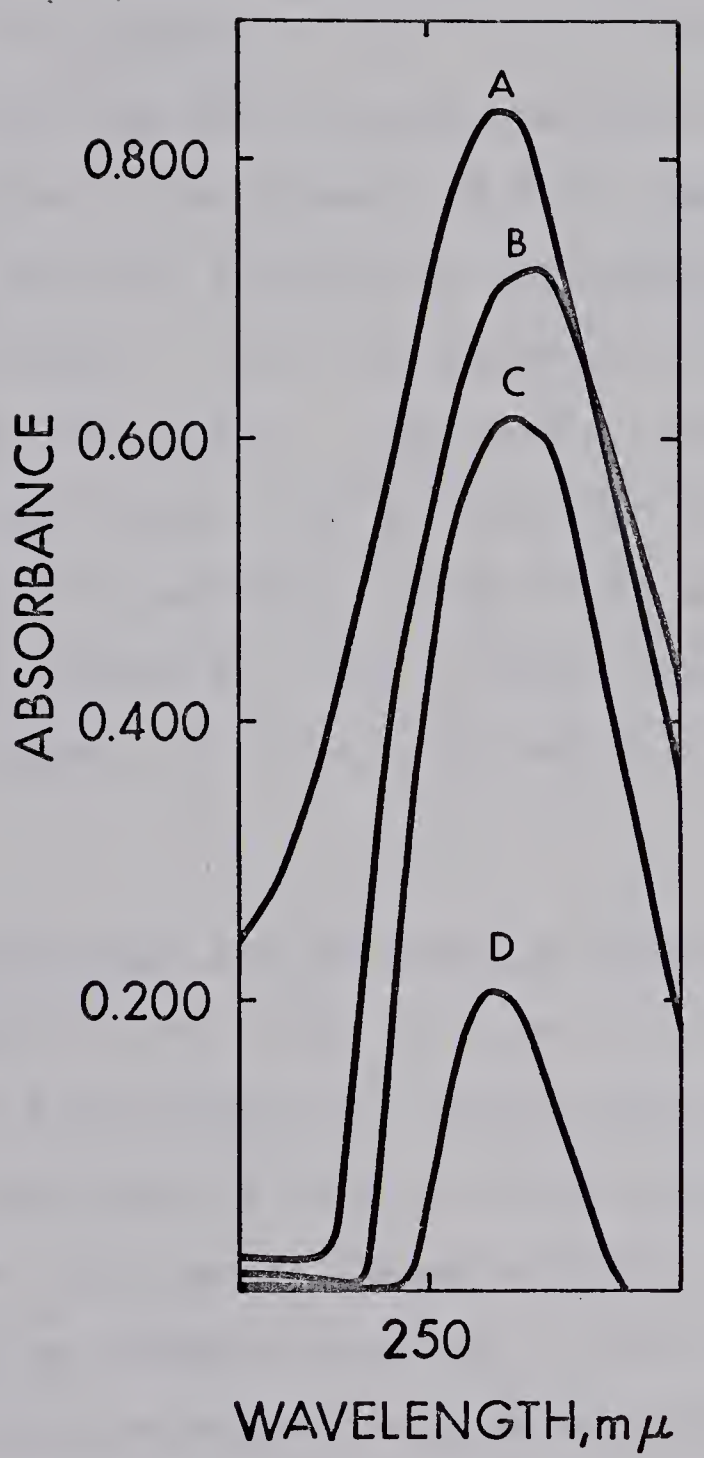

FIG. 15. THE INFLUENCE OF BOVINE SERUM ALBUMIN ON THE SPECTRUM OF BUTYLPARABEN AT pH 7.4.

KEY: A, no BSA; B, 0.025mMBSA; C, 0.05mMBSA; D, 0.1mMBSA.

Spectrophotometric Studies of the Paraben-Protein Interaction

Figures 12 to 15 indicate the influence of BSA on the absorption spectrum of parabens at pH 7.4 with ionic strength 0.16. Curve A was the spectroscopic behavior of a buffered paraben solution in the absence of BSA. The spectra of all parabens were not affected by the presence of components of phosphate buffer. Here, the presence of protein resulted in a hypochromic effect. Absorbance values at 256 mμ were lower in the presence of protein than in protein-free solution for all parabens. This can be seen by comparing curve A with curves B, C and D, which shows the absorbance in the presence of 0.025, 0.05 and 0.1 mM BSA respectively.

Correlation of Binding Data with the Efficacy of Parabens

Earlier studies (26,66) have confirmed that for any given concentration of macromolecules (polysorbate 80 and cetomacrogol 1000) the ratio of total to free preservative is a constant, Re. If the antifungal activity of the parabens studied can be assumed to be due to the free or unbound form, then the Re value can be used to predict the minimum inhibitory concentration of antifungal agent (27,30) in the presence of a definite concentration of BSA in the following manner:

$$Re = \frac{\text{Total paraben}}{\text{Free paraben}}$$

Where Total paraben = Free + Bound form of paraben.

Free paraben = Inhibitory concentration of the paraben in absence of BSA

Thus, Re x Free paraben = Effective inhibitory concentration in the presence of BSA.

This procedure was employed in the present investigation to predict the theoretical or predicted inhibitory concentration of methyl and propylparaben from the binding data.

The Re values for a 0.1 mM BSA solution at pH 6.6 and at 30° for methyl and propylparaben were 1.2 and 1.75 respectively, which were computed as the average of Re values in Table XIX and XX. The minimum inhibitory concentration of methyl and propylparaben for Aspergillus niger in the culture media were found to be 0.060 and 0.020% respectively. These are in good agreement with the values reported previously (30).

Therefore, the theoretical inhibitory concentration of parabens in the presence of 0.1 mM (0.69%) BSA were 0.060% x 1.2 = 0.072% for methylparaben; and 0.020% x 1.75 = 0.035% for propylparaben.

The results of the in vitro correlation of binding data with the inhibitory concentration of methyl and propylparaben for Aspergillus niger in the presence of 0.69% BSA are shown in Tables XXI and XXII. The data show a good correlation between the predicted minimum inhibitory concentration 0.072% and the experimental value of 0.074% for methylparaben;

and the predicted value of 0.035% and the experimental value of 0.035% for propylparaben.

TABLE II

The Binding of Methylparaben by 0.1mM Bovine Serum Albumin at pH 7.4 at 30°

Original Concn. moles/L. x 10^3	Free Concn. (A) moles/L. x 10^3	Total Concn. moles/L. x 10^3	Moles Bound x 10^6	r	r/(A) x 10^{-3}
0.987	0.349	0.655	3.06	3.06	8.78
1.32	0.461	0.850	3.88	3.88	8.42
1.65	0.629	1.04	4.10	4.10	6.52
1.97	0.758	1.22	4.59	4.59	6.05
2.30	0.900	1.43	5.34	5.34	5.94
2.63	1.06	1.62	5.55	5.55	5.24
2.96	1.21	1.79	5.71	5.71	4.70
3.29	1.34	2.02	6.73	6.73	5.02
3.95	1.66	2.35	6.90	6.90	4.15
4.61	2.01	2.70	6.93	6.93	3.45
6.58	2.92	3.75	8.36	8.36	2.87
7.24	3.26	4.12	8.60	8.60	2.64
5.26	2.16	2.82	6.63	6.63	3.07
7.90	3.40	4.22	8.11	8.11	2.38
13.2	5.90	6.93	10.3	10.3	1.75

TABLE III

The Binding of Methylparaben by 0.1mM Bovine Serum Albumin at pH 7.4 and at 10°

Original Concn. moles/L. x 10^3	Free Concn. (A) moles/L. x 10^3	Total Concn. moles/L. x 10^3	Moles Bound x 10^6	r	r/(A) x 10^{-3}
0.987	0.330	0.674	3.44	3.44	10.4
1.32	0.475	0.836	3.60	3.60	7.59
1.65	0.600	1.06	4.65	4.65	7.75
1.97	0.731	1.27	5.39	5.39	7.38
2.63	1.01	1.65	6.40	6.40	6.33
2.96	1.18	1.80	6.20	6.20	5.26
3.29	1.33	1.99	6.47	6.47	4.85
3.62	1.47	2.22	7.55	7.55	5.14
3.95	1.60	2.39	7.82	7.82	4.87
4.61	1.89	2.80	9.16	9.16	4.86
5.26	2.20	3.07	8.69	8.69	3.94
6.58	2.86	3.77	9.03	9.03	3.15
7.24	3.16	4.04	8.76	8.76	2.77
7.90	3.48	4.37	8.89	8.89	2.56
10.5	4.72	5.76	9.61	9.61	2.04
11.8	5.39	6.57	11.8	11.8	2.19

TABLE IV

The Binding of Ethylparaben by 0.1mM Bovine Serum Albumin at pH 7.4 and at 30°

Original Concn. moles/L. x 10^3	Free Concn. (A) moles/L. x 10^3	Total Concn. moles/L. x 10^3	Moles Bound x 10^6	r	r/(A) x 10^{-3}
0.843	0.264	0.561	2.96	2.96	11.2
1.27	0.434	0.818	3.84	3.84	8.85
2.11	0.788	1.31	5.25	5.25	6.67
2.53	0.983	1.56	5.79	5.79	5.89
2.95	1.18	1.79	6.13	6.13	5.20
3.37	1.37	2.05	6.73	6.73	4.90
4.22	1.72	2.46	7.41	7.41	4.32
1.21	0.380	0.731	3.51	3.51	9.22
2.41	0.833	1.38	5.45	5.45	6.55
3.01	1.12	1.75	6.28	6.28	5.63
3.61	1.38	2.09	7.16	7.16	5.20
4.82	1.90	2.74	8.45	8.45	4.46
6.02	2.13	3.45	8.89	8.89	4.18

TABLE V

The Binding of Ethylparaben by 0.1mM Bovine Serum Albumin at pH 7.4 and at 20°

Original Concn. moles/L. x 10^3	Free Concn. (A) moles/L. x 10^3	Total Concn. moles/L. x 10^3	Moles Bound x 10^6	r	r/(A) x 10^{-3}
0.843	0.278	0.623	3.45	3.45	12.4
1.27	0.469	0.880	4.11	4.11	8.76
1.69	0.616	1.19	5.73	5.73	9.30
2.11	0.825	1.42	5.96	5.96	7.22
2.53	1.00	1.74	7.34	7.34	7.32
2.95	1.17	1.99	8.16	8.16	6.99
3.37	1.39	2.19	8.02	8.02	5.78
4.22	1.79	2.66	8.75	8.75	4.90
5.06	2.19	3.15	9.63	9.63	4.40
4.22	1.66	2.54	8.75	8.75	5.26
4.82	1.93	2.90	9.23	9.23	4.68
5.42	2.30	3.33	10.3	10.3	4.48
6.02	2.53	3.58	10.5	10.5	4.16
7.23	3.00	4.05	10.6	10.6	3.53
7.83	3.40	4.50	11.0	11.0	3.24

TABLE VI

The Binding of Propylparaben by 0.1mM Bovine Serum Albumin at pH 7.4 and at 30°

Original Concn. moles/L. $\times 10^{3}$	Free Concn. (A) moles/L. $\times 10^{4}$	Total Concn. moles/L. $\times 10^{4}$	Moles Bound $\times 10^{6}$	r	r/(A) $\times 10^{-3}$
0.556	1.24	4.21	2.97	2.97	23.9
0.833	2.08	6.18	4.10	4.10	19.7
1.11	2.64	8.18	5.54	5.54	21.0
1.39	3.52	10.2	6.67	6.67	18.9
1.67	4.74	12.0	7.28	7.28	15.4
1.95	5.88	13.8	8.00	8.00	13.6
2.22	6.18	15.3	8.48	8.48	12.5
2.50	8.35	17.0	8.62	8.62	10.3
2.78	9.49	18.6	9.09	9.09	9.58
3.06	10.9	20.0	9.19	9.19	8.44
3.33	11.8	21.7	9.96	9.96	8.48
3.61	13.4	23.8	10.4	10.4	7.80
3.89	15.0	25.2	10.2	10.2	6.65
4.17	16.1	25.3	9.22	9.22	5.73
4.44	17.2	26.9	9.75	9.75	5.70
4.72	18.0	28.2	10.2	10.2	5.63
5.00	19.5	30.4	10.9	10.9	5.58
1.39	3.65	9.72	6.08	6.08	16.6
1.67	4.48	11.4	6.90	6.90	15.4
1.95	5.80	13.1	7.34	7.34	12.7
2.22	7.08	14.9	7.85	7.85	11.1
2.50	7.81	16.5	8.69	8.69	11.1
2.18	9.17	18.2	8.99	8.99	9.80

TABLE VII

The Binding of Propylparaben by 0.1mM Bovine Serum Albumin at pH 7.4 and at 20°

Original Concn. moles/L. x 10^3	Free Concn. (A) moles/L. x 10^4	Total Concn. moles/L. x 10^4	Moles Bound x 10^6	r	r/(A) x 10^{-3}
0.833	1.72	6.59	4.67	4,67	24.4
1.11	2.79	8.93	6.14	6.14	22.0
1.39	3.62	10.7	7.05	7.05	19.4
1.67	4.71	12.6	7.90	7.90	16.8
1.95	5.59	14.5	8.88	8.88	15.9
2.22	6.88	16.1	9.23	9.23	13.4
2.50	8.12	18.0	9.87	9.87	12.1
2.78	9.06	19.8	10.7	10.7	12.9
3.06	10.4	21.7	11.3	11.3	10.9
3.33	11.4	23.0	11.6	11.6	10.2
3.36	12.9	24.8	11.9	11.9	9.27
2.50	7.62	17.8	10.2	10.2	13.8
2.78	8.76	18.6	9.87	9.87	11.3
3.06	10.3	20.9	10.7	10.7	10.4
3.33	11.4	22.3	10.9	10.9	9.62
3.61	11.3	22.5	11.1	11.1	9.82
3.89	13.8	25.4	11.6	11.6	8.44
4.17	15.2	27.0	11.8	11.8	7.75

TABLE VIII

The Binding of Propylparaben by 0.25 mM Bovine Serum Albumin at pH 7.4 and at 30°

Original Concn. moles/L. $\times 10^3$	Free Concn. (A) moles/L. $\times 10^4$	Total Concn. moles/L. $\times 10^4$	Moles Bound $\times 10^6$	r	$r/(A)_{-3}$ $\times 10^{-3}$
0.833	1.01	7.65	6.64	2.66	26.4
1.11	1.51	10.7	9.23	3.69	24.4
1.39	1.98	13.2	11.2	4.47	22.6
1.67	2.58	15.6	13.1	5.22	20.2
1.95	3.22	17.9	14.7	5.88	18.2
2.22	3.78	19.8	16.0	6.41	17.0
2.50	4.63	22.3	17.7	7.09	15.3
2.78	5.23	24.3	19.1	7.62	14.6
3.06	5.97	25.1	19.1	7.65	12.8
3.33	6.91	27.4	20.5	8.19	11.8
3.61	7.92	29.0	21.1	8.44	10.7
3.89	8.79	32.0	23.3	9.30	10.6
4.17	9.90	32.4	22.5	8.99	9.08
4.44	10.9	33.9	23.0	9.20	8.43

TABLE IX

The Binding of Butylparaben by 0.1 mM Bovine Serum Albumin at pH 7.4 and at 30°

Original Concn. moles/L. x 10^4	Free Concn. (A) moles/L. x 10^4	Total Concn. moles/L. x 10^4	Moles Bound x 10^6	r	r/(A) x 10^{-4}
5.15	0.583	4.54	3.95	3.95	6.78
6.19	0.778	5.35	4.57	4.57	5.88
7.22	0.959	6.12	5.17	5.17	5.38
8.25	1.26	6.80	5.54	5.54	4.38
9.28	1.52	7.22	5.70	5.70	3.74
10.3	1.77	7.94	6.17	6.17	3.50
11.3	2.06	8.46	6.40	6.40	3.11
12.4	2.44	9.43	7.00	7.00	2.87
13.4	2.72	10.1	7.36	7.36	2.70
15.5	2.42	10.8	7.40	7.40	2.17
16.5	3.84	11.5	7.70	7.70	2.00
17.5	4.25	12.1	7.81	7.81	1.84
18.6	4.63	13.8	9.19	9.19	1.98
7.73	1.00	5.81	4.81	4.81	4.80
9.28	1.37	6.99	5.62	5.62	4.09
10.8	1.82	7.99	6.17	6.17	3.40
12.4	2.23	8.66	6.43	6.43	2.88
13.1	2.54	9.44	6.92	6.92	2.74
13.9	2.76	9.77	7.01	7.01	2.54

TABLE X

The Binding of Butylparaben by 0.1mM Bovine Serum Albumin at pH 7.4 and at 20°

Original Concn. moles/L. x 10^4	Free Concn, (A) moles/L. x 10^4	Total Concn. moles/L. x 10^4	Moles Bound x 10^6	r	r/(A) x 10^{-4}
5.15	0.544	4.60	4.06	4.06	7.45
6.19	0.684	5.44	4.76	4.76	6.96
7.22	0.875	6.37	5.49	5.49	6.28
8.25	1.10	7.21	6.11	6.11	5.58
9.28	1.36	8.04	6.68	6.68	4.90
10.3	1.69	8.91	7.23	7.23	4.29
11.3	1.93	9.62	7.70	7.70	4.99
12.4	2.29	10.2	8.00	8.00	3.48
13.4	2.74	11.1	8.38	8.38	3.06
14.4	2.93	12.2	9.25	9.25	3.15
15.5	3.40	12.8	9.36	9.36	2.75
16.6	3.69	13.3	9.59	9.59	2.60
17.5	4.16	13.9	9.77	9.77	2.35
18.6	4.50	14.5	9.95	9.95	2.21
8.76	1.10	7.58	6.48	6.48	5.88
9.28	1.25	8.17	6.92	6.92	5.53
9.79	1.37	8.55	7.19	7.19	5.25
10.3	1.45	8.90	7.45	7.45	5.16
10.8	1.65	8.77	7.12	7.12	4.33
11.3	1.79	9.61	7.82	7.82	4.37
11.9	1.93	9.80	7.89	7.89	4.09
12.4	2.15	10.4	8.21	8.21	3.82
12.9	2.32	10.5	8.29	8.29	3.57
13.4	2.54	11.0	8.48	8.48	3.34

TABLE XI

Binding Constants and Thermodynamic Functions of Paraben-Protein Interaction in Aqueous Solution at pH 7.4

Paraben	Temperature	Binding Constant (k) L/mole x 10^{-3}	nk x 10^{-3}	No. of Sites n	Free Energy change (ΔF^{o}) kcal/mole	Enthalpy change (ΔH^{o}) kcal/mole	Entropy change (ΔS^{o}) e.u.
Methyl	30^{o}	0.987	10.9	11.2	-4.15	0.0845	14.0
	10^{o}	0.968	12.1	12.5	-3.87		
Ethyl	30^{o}	1.05	12.8	12.2	-4.19	0.529	15.6
	20^{o}	1.02	14.4	14.1	-4.04		
Propyl	30^{o}	2.33	31.3	13.4	-4.67	0.876	18.3
	20^{o}	2.22	34.9	15.7	-4.99		
Butyl	30^{o}	10.1	99.4	9.9	-5.56	4.00	31.6
	20^{o}	8.11	105	12.9	-5.24		

TABLE XII

The Binding of Propylparaben by Various Concentrations of Bovine Serum Albumin at pH 7.4 and at 30°

(Initial concentration of propylparaben = 2.63×10^{-3} M)

BSA Concn. m moles/L.	Free Concn. moles/L. $\times 10^{3}$	Total Concn. moles/L. $\times 10^{3}$	Moles Bound $\times 10^{6}$	Per cent Bound
0.02	1.26	1.35	0.881	6.53
0.04	1.18	1.49	3.05	20.6
0.06	1.06	1.56	5.03	32.2
0.08	1.00	1.68	6.82	40.5
0.1	0.917	1.82	8.99	49.5

TABLE XIII

The Solubility of Hexylparaben in Various Concentrations of Bovine Serum Albumin at pH 7.4 and at 30°

BSA Concn. moles/L. x 10^5	Concn. of Hexyl Paraben moles/L. x 10^4	R_s^*
0	2.80	
1.00	4.23	1.51
2.00	5.91	2.11
3.00	6.73	2.40
4.00	7.51	2.68
5.00	8.87	3.17
6.00	9.33	3.33
7.00	10.9	3.89
8.00	12.7	4.54
9.00	13.2	4.71
10.0	13.8	4.93

$$R_s^* = \frac{\text{Solubility in Buffered BSA Solution}}{\text{Solubility in Buffered Aqueous Solution}}$$

TABLE XIV

The Solubility of Heptylparaben in Various Concentrations of Bovine Serum Albumin at pH 7.4 and at 30°

BSA Concn, moles/L. x 10^5	Concn. Heptylparaben moles/L. x 10^4	R_s^*
0	0.708	
1.00	1.90	2.68
2.00	2.57	3.63
3.00	3.98	5.62
4.00	4.34	6.13
5.00	5.09	7.19
6.00	6.24	8.81
7.00	7.30	10.3
8.00	8.32	11.8
9.00	8.76	12.4
10.00	10.4	14.7

$$R_s^* = \frac{\text{Solubility in Buffered BSA Solution}}{\text{Solubility in Buffered Aqueous Solution}}$$

TABLE XV

The Binding of Methylparaben as a Function of pH at 30°

Initial Concentration of Methylparaben = 2.63×10^{-3}M

BSA = 1.0×10^{-4}M

pH	Free Concn. (A) moles/L. $\times 10^{3}$	Total Concn. moles/L. $\times 10^{3}$	Moles Bound $\times 10^{6}$	r	r/(A) $\times 10^{-3}$
4.30	1.17	1.51	3.37	3.37	2.87
5.05	1.12	1.48	3.57	3.57	3.19
6.00	1.11	1.54	4.29	4.29	3.86
6.90	1.13	1.55	4.30	4.30	3.80
7.20	1.11	1.54	4.33	4.33	3.90
7.30	1.10	1.55	4.49	4.49	4.08
7.40	1.11	1.56	4.56	4.56	4.11
7.60	1.10	1.15	4.42	4.42	3.98
7.80	1.06	1.48	4.21	4.21	4.00
8.00	1.13	1.54	4.13	4.13	3.65
8.45	1.12	1.50	3.88	3.88	2.46
8.90	1.14	1.43	2.90	2.90	2.55

TABLE XVI

The Binding of Ethylparaben as a Function of pH at 30°

Initial Concentration of Ethylparaben = 2.41×10^{-3}M

BSA = 1.0×10^{-4}M

pH	Free Concn.(A) moles/L. $\times 10^{3}$	Total Concn. moles/L. $\times 10^{3}$	Moles Bound $\times 10^{6}$	r	r/(A) $\times 10^{-3}$
4.20	1.03	1.45	4.17	4.17	4.04
5.10	1.00	1.44	4.38	4.38	4.36
6.10	0.933	1.44	5.08	5.08	5.45
7.00	0.896	1.46	5.52	5.52	6.17
7.20	0.896	1.44	5.46	5.46	6.09
7.60	0.897	1.45	5.69	5.69	6.48
8.20	0.923	1.48	5.59	5.59	6.06
8.40	0.913	1.47	5.56	5.56	6.09
8.90	0.956	1.40	4.41	4.41	4.61

TABLE XVII

The Binding of Propylparaben as a Function of pH at 30°

Initial Concentration of Propylparaben = 2.22×10^{-3}M

BSA = 1.0×10^{-4}M

pH	Free Concn. (A) moles/L. $\times 10^{4}$	Total Concn. moles/L. $\times 10^{3}$	Moles Bound $\times 10^{6}$	r	r/(A) $\times 10^{-3}$
4.10	8.05	1.32	5.17	5.17	6.42
5.05	7.99	1.37	5.70	5.70	7.14
6.05	7.65	1.40	6.38	6.38	8.33
7.00	7.21	1.48	7.55	7.55	10.5
7.40	7.38	1.54	8.05	8.05	10.9
7.50	7.38	1.57	8.32	8.32	11.3
7.65	6.85	1.47	7.85	7.85	11.5
8.05	7.21	1.54	8.22	8.22	11.4
8.25	7.32	1.51	7.79	7.79	10.6
8.50	7.45	1.45	7.08	7.08	9.50
8.70	7.58	1.46	6.98	6.98	9.20
8.95	7.85	1.42	6.38	6.38	8.12

TABLE XVIII

The Binding of Butylparaben as a Function of pH at 30°

Initial Concentration of Butylparaben = 1.03×10^{-4}M

BSA = 1.0×10^{-4}M

pH	Free Concn. (A) moles/L. $\times 10^4$	Total Concn. moles/L. $\times 10^4$	Moles Bound $\times 10^6$	r	$r/(A)_4 \times 10$
4.15	2.69	7.24	4.55	4.55	1.69
5.00	2.53	6.82	4.29	4.29	1.70
5.95	2.18	7.05	4.87	4.87	4.23
6.80	1.83	7.60	5.77	5.77	3.15
7.10	1.66	7.69	6.03	6.03	3.63
7.30	1.58	7.21	5.63	5.63	3.56
7.45	1.54	7.27	5.73	5.73	3.72
7.80	1.51	7.18	5.67	5.67	3.75
8.35	1.54	7.05	5.51	5.51	3.58
8.90	2.14	6.95	4.81	4.81	2.25

TABLE XIX

The Binding of Methylparaben by 0.1 mM Bovine Serum Albumin at pH 6.6 and at 30°

Original Concn. %	Free Concn. moles/L. x 10^3	Total Concn. moles/L. x 10^3	Moles Bound x 10^6	$R_e = \frac{\text{Total}}{\text{Free}}$
0.11	3.40	4.08	6.81	1.20
0.12	3.67	4.51	8.43	1.23
0.13	4.00	4.89	8.89	1.22
0.14	4.29	5.19	9.03	1.21
0.15	4.72	5.59	8.76	1.19
0.16	5.01	6.05	10.4	1.21
0.17	5.39	6.27	8.86	1.16
0.18	5.51	6.52	10.1	1.18
0.19	6.01	7.00	9.94	1.17
0.20	6.32	7.31	9.94	1.16

TABLE XX

The Binding of Propylparaben by 0.1 mM Bovine Serum Albumin at pH 6.6 and at 30°

Original Concn. %	Free Concn. moles/L. $\times 10^4$	Total Concn. moles/L. $\times 10^4$	Moles Bound $\times 10^6$	$R_e = \frac{Total}{Free}$
0.045	8.56	16.7	8.15	1.95
0.050	9.73	18.3	8.52	1.88
0.055	11.1	20.4	9.30	1.84
0.060	12.3	22.5	10.2	1.83
0.065	13.4	23.4	9.93	1.74
0.070	13.5	23.6	10.2	1.75
0.075	15.7	26.6	10.8	1.69
0.080	17.1	27.6	10.5	1.61
0.085	18.0	29.5	11.5	1.64
0.090	19.3	30.9	11.5	1.60

TABLE XXI

Growth Studies of Aspergillus Niger in 0.69% Bovine Serum Albumin with Various Concentrations of Methylparaben

Methylparaben Concentration %	Growth After 7 Days
0.060	+
0.070	+
0.072	+
0.074	+
0.076	-
0.078	-
0.080	-
0.090	-
0.100	-

+ = growth

- = no growth

TABLE XXII

Growth Studies of Aspergillus Niger in 0.69% Bovine Serum Albumin with Various Concentrations of Propylparaben

Propylparaben Concentration %	Growth After 7 Days
0.020	+
0.025	+
0.030	+
0.035	-
0.040	-
0.045	-

+ = growth

- = no growth

DISCUSSION

The ability of p-hydroxybenzoates to interact with several macromolecules has been well documented (26,28,30). Based upon this observation, it was expected that BSA might provide binding sites for the parabens. From Figures 1 to 7, it is evident that all the parabens interacted with BSA. The values of n are not integers (Table XI). This might be either due to the experimental error as explained by Klotz (70) or due to the fact that protein might not be molecularly homogeneous with respect to binding properties as theorized by Karush (67).

The exact mechanism of binding of p-hydroxybenzoates by BSA can not be elucidated from this study, since the results are obtained primarily from the binding of methyl, ethyl, propyl and butylparaben. However, certain tentative conclusions may be drawn. A comparison of binding constants for various parabens at a definite temperature (Table XI) reveals that the binding increases from methyl to ethyl to propyl and to butylparaben. Although no binding constants were computed for hexyl and heptylparabens, the ratio of total/free drug as obtained from the solubility study for these parabens can be employed as a qualitative measure of binding (26), for the purpose of comparing with similar values obtained from the dialysis data of the other parabens.

The values of these ratios for methyl, ethyl, propyl, butyl, hexyl and heptylparaben are 1.2*, 1.4*, 1.6*, 2.8*, 4.9 and 14.7 respectively, thus demonstrating that the binding increases as size of the non-polar aliphatic side chain in the paraben increases. About 40% of the total amino acids in the protein (62) contains non-polar side chains and it is quite possible that the association between these chains and the non-polar side chain in the parabens may contribute the driving force for interaction. An examination of the data of Table XI shows that the stability of the complex formed between BSA and parabens increased with increase in temperature demonstrating that the binding process was endothermic (unfavorable positive enthalpy). More significantly the binding was associated with an increase in entropy which is typically a thermodynamic behavior in common with that described for hydrophobic bonding (71). A similar thermodynamic property of parabens and other small molecules for polyvinylpyrrolidone has been well substantiated in the literature (72,73).

The increase in the positive enthalpy term, ΔH^{o}, due to increase in aliphatic side chain in the paraben may have overshadowed any possible negative enthalpy contribution made by hydrogen bond formation between -OH and C=O groups of parabens and COO^{-}, -OH and C=O groups of the

*These values were obtained from Table II, IV, VI and IX by appropriate computation, and the calculated value represents the average of the highest four initial drug concentrations in the system.

protein. It can be noted in Table XI that ΔH^{o} values for methyl and butylparabens are 0.0845 and 4.00 kcal/mole. The polarity of the -OH group in paraben molecules decreases with an increase in the ester group, which in turn lowers their ability to form hydrogen bonds (73).

The pH profile on paraben-protein binding as illustrated in Figures 8 to 11 demonstrates that the interaction is pH sensitive. The increase in interaction from pH 4 until a maximum was achieved at pH 7.6 $\pm$ 0.2 can be attributed to the ionization of parabens. The pka values of methyl, ethyl, propyl and butylparaben are 8.0 to 8.2 (74). The fraction of paraben in the anionic form increases as pH value increases. It appears that the negatively charged form is more strongly bound than the uncharged form. The marked decrease in binding above pH 7.6 $\pm$ 0.2 may be explained either by assuming that a specific interaction occurred between parabens and protonated sites on the paraben or by considering that the binding site involved was either blocked or abolished by configurational change which resulted from the neutralization of protonated groups on the protein molecule (10).

Figures 8 to 11 further indicate that parabens were bound to a certain extent in the pH region of 4 to 6. This may be due to hydrogen bond formation as explained in the previous paragraph. The pH dependency of the interaction in this pH range, where parabens were primarily in their undissociated form, is similar to the observation

made by Klotz and Ayers (75). They showed that neutral molecules such as aminobenzene were bound more strongly as the pH was raised. They attributed this phenomenon to the availability of new binding sites upon increase in pH.

In Figures 12 to 15, the degree of hypochromism increases with an increase in concentration of protein. This displacement has been interpreted by Klotz (51) as an effect due to the formation of drug-protein complex, if the complexation involves a chromophor system of drug. Although spectrophotometric study can often yield information regarding magnitude of the interaction as well as the nature of forces responsible for the interaction, this study was limited to the qualitative nature of the interaction.

The results of the *in vitro* correlation of binding data with the inhibitory concentration of methyl and propylparaben for *Aspergillus niger* in the presence of 0.69% BSA are excellent. The ratio Re of total/free paraben can be used to predict the minimum inhibitory concentration of the antifungal agents in the presence of a definite concentration of protein. This part of the study demonstrates that the bound form of the drug is devoid of *in vitro* biological activity.

No attempt was made to correlate the binding data with *in vivo* studies. However, it is probable that there is a direct relationship between the magnitude of paraben bind-

ing and biological half-life of the parabens. By means of urinary excretion data, Jones et al. (76) showed that the biological half-lives of parabens were as follows: methylparaben, 14 hours; ethylparaben, 18 hours; propylparaben, 22 hours and butylparaben 55 hours. It is to be expected that the biological half-life would increase as the binding of paraben with protein increases.

SUMMARY

1. The binding of a series of p-hydroxybenzoates by bovine serum albumin has been investigated by equilibrium dialysis, solubility and spectrophotometric techniques.
2. The degree of binding increased from methyl, to ethyl to propyl to butyl to hexyl to heptylparaben. The relative binding affinities were obtained graphically and free energy change, ΔF°; enthalpy change, ΔH° and entropy change, ΔS° were computed. The contribution of hydrophobic bonding to the paraben-protein interaction has been discussed.
3. The pH profile on paraben-protein interaction has been studied.
4. Spectrophotometric studies on the paraben-protein interaction showed that there was a hypochromic effect due to the interaction, and the magnitude of hypochromism in the case of binding of methyl, ethyl, propyl and butylparaben increased as the concentration of BSA was increased.
5. It was demonstrated by an <u>in vitro</u> microbiological procedure that the antifungal activity of methyl and propylparaben was primarily a function of free drug.

BIBLIOGRAPHY

1. Goldstein, A., Pharmacol. Revs., 1, 102 (1949).

2. Klotz, I.M., In "The Proteins," Vol. 1, Part B, Edited by Neurath, H., and Bailey, K., Academic Press, Inc., New York, N.Y., 1953, p. 727.

3. Klotz, I.M., In "Molecular Structure of Biological Specificity," Edited by Pauling, L., and Itano, H. A., American Institute of Biological Science, Washington, D.C., 1957, p. 111.

4. Molyneux, P., and Frank, H.P., J. Am. Chem. Soc., 83, 3169 (1961).

5. Lindenbaum, A., and Schubert, J., J. Phys. Chem., 60, 1663 (1956).

6. Davison, C., and Smith, P.K., J. Pharmacol. Exptl. Therap., 133, 161 (1961).

7. Wohler, F., and Speckmann, L., Arzneimittel - Forsch., 10, 859 (1960).

8. Anton, A.N., J. Pharmacol. Exptl. Therap., 129, 282 (1960).

9. Klotz, I.M., Urquhart, J., and Weber, W., Arch. Biochem., 26, 420 (1950).

10. Eichman, M.L., Guttman, D.E., Van Winkle, Q., and Guth, E.P., J. Pharm. Sci., 51, 66 (1962).

11. Brodie, B.B., and Hogben, C.A.A., J. Pharm. Pharmacol., 9, 345 (1957).

12. Goldblum, R.W., Piper, W.N., and Olson, C.J., J. Invest. Dermatol., 25, 139 (1955); Chem. Abstr., 51, 1459a (1957).

13. Puttnam, N.A., J. Chem. Soc., 38, 486 (1960).

14. Patel, N.K., and Murray, J.D., unpublished data.

15. Boyer, P.D., Ballou, G.A., and Luck, J.M., J. Biol. Chem., 167, 407 (1947).

16. Mann, R.L., In "Reminton's Pharmaceutical Sciences," 13th Ed., Edited by Martin, E.W., Mack Publishing Co., Easton, Pa., 1965, p.1228.

17. Smith, R.F., Shay, D.E., and Doorenbos, F.L., J. Appl. Microbiol., 11, 542 (1963).

18. Wolf, P.A., and Westveer, W.M., Arch. Biochem., 18, 205 (1950).

19. Aalto, M.C., Firman, M.C., and Risler, N.E., J. Am. Pharm. Assoc., Sci. Ed., 42, 449 (1953).

20. Patel, N.K., and Taylor, K.E., Unpublished data.

21. Moore, B., and Roaf, H.E., Proc. Roy, Soc., London, 73, 382 (1904).

22. Moore, B., and Roaf, H.E., ibid., 77, 86 (1905).

23. Davis, B.D., J. Clin. Invest., 22, 753 (1943).

24. Tompsett, R., Shultz, S., and McMermott, W., J. Bacteriol., 53, 581 (1947).

25. Quinn, E.L., Paper presented to the conference on therapy with the new penicillins, held at the Apothecaries Hall, London, June, 1964.

26. Patel, N.K., and Kostenbauder, H.B., J. Am. Pharm. Assoc., Sci. Ed., 47, 289 (1958).

27. Pisano, F.D., and Kostenbauder, H.B., ibid., 48, 310 (1959).

28. Miyawaki, G.M., Patel, N.K., and Kostenbauder, H.B., ibid., 48, 315 (1959).

29. Deluca, P.P., and Kostenbauder, H.B., ibid., 49, 430 (1960).

30. Patel, N.K., Can. J. Pharm. Sci., in Press.

31. Martin, A.N., "Physical Pharmacy," Lea and Febiger, Philadelphia, Pa., 1960, p. 425.

32. Kunin, C.M., J. Lab. Clin. Med., 65, 416 (1965).

33. Throp, J.M., In "Absorption and Distribution of Drugs," Edited by Binns, T.B., E. & S. Livingstone Ltd., London, 1964, p. 64.

34. Smith, P.K., Gleason, H.L., Stoll, C.M., and Ogorzalek, S., J. Pharmacol. Exptl. Therap., 80, 193 (1944).

35. Monaco, F., Gazz, Intern. Med. Chir., 70, 763 (1965); Chem. Abstr., 51, 1459a (1957).

36. Yoshioka, O., Inomata, Y., and Hakazawa, S., J. Antibiotics (TOKYO), 18, 17 (1965); Chem. Abstr., 62, 11425g, (1965).

37. Kivman, G.Y., and Profinjeva, R.P., Vop. Med. Khim, 10, 402 (1964); Chem. Abstr., 62, 9640a (1965).

38. Daughaday, W.H., Physiol. Revs., 39, 885 (1959).

39. Eagle, H., and Newman, E., J. Clin. Invest., 26, 903 (1947).

40. Sisota, J.H., Saltzman, A., and Warmbrand, H., J. Pharmacol. Exptl. Therap., 100, 210 (1950).

41. Heymann, F., and Fieser, L.F., ibid., 94, 97 (1948)

42. Sahyun, M.R.V., Nature, 203, 1045 (1964).

43. Sahyun, M.R.V., ibid., 209, 613 (1966).

44. Klotz, I.M., and Walker, F.M., J. Am. Chem. Soc., 69, 1609 (1947).

45. Westphal, U., Ashley, B.D., and Seldon, G.L., ibid., 80, 5135 (1958).

46. Guttman, D.E., and Gadzala, A.E., J. Pharm. Sci., 54, 742 (1965).

47. Stromme, J.H., Biochem. Pharmacol., 14, 381 (1965).

48. Scatchard, G., Scheinberg, I.H., and Armstrong, S.H., J. Am. Chem. Soc., 72, 540 (1950).

49. Efimenko, A.M., Farmakol. Toksikol., 27, 537 (1964); Chem. Abstr., 62, 5757h (1965).

50. Osborne, W.A., J. Physiol., 34, 84 (1906).

51. Klotz, I.M., J. Am. Chem. Soc., 68, 2299 (1946).

52. Klotz, I.M., Science, 128, 815 (1958).

53. Klotz, I.M., Burkhard, R.K., and Urquhart, J.M., J. Am. Chem. Soc., 74, 202 (1952).

54. Yanari, S., and Bovey, F.A., J. Biol. Chem., 235, 2818 (1960).

55. Luck, J.M., and Schmit, A.S., Stanford Med. Bull., 6, 133 (1948).

56. Bjorntorp, P., Ells, H.A., and Brandford, R.H., J. Biol. Chem., 239, 339 (1964).

57. Saroff, H.A., and Lewis, M.S., J. Phys. Chem., 67, 1211 (1963).

58. Hermans, J.Jr., Leach, S.J., and Scheraga, H.A., J. Am. Chem. Soc., 85, 1390 (1962).

59. Klotz, I.M., and Urquhart, J.M., ibid., 71, 847 (1949).

60. Waugh, D.F., Advan. Protein Chem., 9, 325 (1954).

61. Scheraga, H.A., Nemethy, G., and Steinberg, 1., J. Biol. Chem., 237, 2506 (1962).

62. Nemethy, G., and Scheraga, H.A., J. Phys. Chem., 66, 1773 (1962).

63. Steinhard, J., and Beychok, S., In "The Proteins," Vol. II, Edited by Neurath, H., Aacademic Press, New York, N.Y., 1964, p. 283.

64. Kauzman, W., In "The Mechanism of Enzyme Action," Edited by McElroy, W.D., and Glass, B., Johns Hopkins University Press, Baltimore, Md., 1954, p. 70.

65. Cohn, E.J., Hughes, W.L., and Weare, J.H., J. Am. Chem. Soc., 69, 1753 (1947).

66. Patel, N.K., and Foss, N.E., J. Pharm. Sci., 54, 1495 (1965).

67. Karush, F., J. Am. Chem. Soc., 72, 2705 (1950).

68. Scatchard, G., Ann. N.Y. Acad. Sci., 51, 660 (1949).

69. Neurath, H., and Schwert, G.W., Chem. Revs., 46, 69 (1950).

70. Klotz, I.M. Walker, F.M., and Pivan, R.B., J. Am. Chem. Soc., 68, 1486 (1946).

71. Schachman, H.K., Cold Spring Habor Sym. Quant. Biol., 28, 409 (1963).

72. Frank. H.P., and Molyneux, P., J. Am. Chem. Soc., 83, 3169 (1961).

73. Jurgensen, G., and Speiser, P., Acta Pharm. Suecica, 4, 185 (1967).

(74) Patel, N.K., unpublished data.

(75) Klotz, I.M., and Ayers, J., J. Am. Chem. Soc., 74, 6178 (1952).

(76) Johnes, P.S., Thigpen, D., Morrison, J.L., and Richardson, A.P., J. Am. Pharm. Assoc., Sci. Ed., 45, 268 (1956).